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Photochemical Rescue of a Conformationally Inactivated Ribonucleotide Reductase

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Abstract

Class Ia ribonucleotide reductase (RNR) of *Escherichia coli* contains an unusually stable tyrosyl radical cofactor in the β_2 subunit ($Y_{122}^\bullet$) necessary for nucleotide reductase activity. Upon binding the cognate α_2 subunit, loaded with nucleoside diphosphate substrate and an allosteric/activity effector, a rate determining conformational change(s) enables rapid radical transfer (RT) within the active $\alpha_2\beta_2$ complex from the $Y_{122}^\bullet$ site in β_2 to the substrate activating cysteine residue (C_{439}) in α_2 via a pathway of redox active amino acids ($Y_{122}[\beta] \leftrightarrow W_{48}[\beta] \leftrightarrow Y_{356}[\beta] \leftrightarrow Y_{731}[\alpha] \leftrightarrow Y_{730}[\alpha] \leftrightarrow C_{439}[\alpha]$) spanning >35 Å. Ionizable residues at the $\alpha_2\beta_2$ interface are essential in mediating RT, and therefore control activity. One of these mutations, $E_{350}X$ (where $X = A, D, Q$) in β_2 , obviates all RT, though the mechanism of control by which E_{350} mediates RT remains unclear. Herein, we utilize an $E_{350}Q$ -photo β_2 construct to photochemically rescue RNR activity from an otherwise inactive construct, wherein the initial RT event ($Y_{122}^\bullet \rightarrow Y_{356}$) is replaced by direct photochemical radical generation of $Y_{356}^\bullet$. These data present compelling evidence that E_{350} conveys allosteric information between the α_2 and β_2 subunits facilitating conformational gating of RT that specifically targets $Y_{122}^\bullet$ reduction, while the fidelity of the remainder of the RT pathway is retained.

Graphical Abstract

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

ASSOCIATED CONTENT

Supporting Information.

Supplemental methods, emission lifetime traces and associated exponential fits, pH dependent photochemical turnover assays for $E_{350}Q:Y_{356}F_2Y$ -photo β_2 , $E_{350}Q:Y_{356}F_2Y$ -photo β_2 [Re^I]* emission lifetimes and $F_2Y_{356}^\bullet$ decay lifetimes. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The authors declare no competing financial interest.

Introduction

Ribonucleotide reductases (RNRs) catalyze the reduction of all four nucleoside diphosphates (adenosine, A; uridine, U; guanosine, G; cytidine, C) to 2'-deoxynucleoside diphosphates that, subsequent to their rapid phosphorylation and metabolism of deoxycytidine triphosphate (dCTP)/deoxyuridine triphosphate (dUTP) to thymine triphosphate (TTP), provide the monomeric precursors in *de novo* DNA biosynthesis and repair.^{1,2} Nucleotide reduction requires activation of the substrate, achieved by abstraction of its 3'-H by a transiently generated thiyl radical, localized on a conserved cysteine residue.^{3–5} In the class I RNRs, formation of the thiyl radical in the active site harboring subunit (α) is controlled by a second subunit (β) containing a redox cofactor, the nature of which forms the basis for subclass differentiation among RNRs.^{1,6,7}

The active $\alpha_2\beta_2$ complex of class Ia RNR of *Escherichia coli* is a paradigm among this enzyme class.^{8–10} The α_2 subunit contains the catalytic site, the thiyl radical forming cysteine residue (C₄₃₉), a nucleoside diphosphate (NDP) substrate binding site, a specificity site that binds (deoxy)nucleosides triphosphates (NTP/dNTP) and controls which NDP is reduced, and an activity site that binds adenosine triphosphate (ATP) or deoxyadenosine triphosphate (dATP), whose ratio governs the overall rate of the reaction.⁹ The β_2 subunit contains a stable diiron-tyrosyl radical cofactor ($\text{Fe}_2^{\text{III}}\text{-O/Y}_{122}\bullet$) essential for C₄₃₉• generation in $\alpha_2 > 35$ Å away.^{11–13} The significant distance between Y₁₂₂•[β] and C₄₃₉[α], as well as the dramatically altered pK_a of the one electron oxidized amino acid sidechains, necessitates long range proton-coupled electron transfer (PCET) to achieve radical transfer (RT).¹³ This long range PCET process involves an intermediary radical “hopping” pathway composed of redox active aromatic amino acids (Y₁₂₂[β] $\leftrightarrow$ W₄₈[β] $\leftrightarrow$ Y₃₅₆[β] $\leftrightarrow$ Y₇₃₁[α] $\leftrightarrow$ Y₇₃₀[α] $\leftrightarrow$ C₄₃₉[α], Fig.1). The active $\alpha_2\beta_2$ complex of Figure 1 forms in the presence of the appropriate substrate and effector pair. A conformational change(s) subsequent to $\alpha_2\beta_2$ formation initiates Y₁₂₂[β]• reduction by an electron from Y₃₅₆[β], and an attendant transfer of a committed proton, which is proposed to come from Fe^{1,14} in this orthogonal PCET event. Currently no evidence exists for the involvement of W₄₈[β] in RT,

despite evidence for its role in metallo-cluster assembly.¹⁵ The resulting $Y_{356}[\beta]\bullet$ is transferred across the subunit interface into α_2 by way of $Y_{731}[\alpha]$, via an orthogonal PCET mechanism. Once $Y_{731}[\alpha]\bullet$ is generated, RT within α_2 , occurs by a collinear mechanism in which proton and electron are derived from the same donor. The thiyl radical initiates NDP reduction and is regenerated during turnover. Reverse RT from $C_{439}[\alpha]\bullet$ to $Y_{122}[\beta]$ completes the catalytic cycle. Despite extensive study, acquiring detailed insight into the RT pathway has been challenging due to the lack of an atomic resolution structure of the $\alpha_2\beta_2$ complex and unresolved residues essential to activity in the available structures of the respective subunits: the $Y_{356}[\beta]$ required for RT^{8,11} and the C-terminal tail of β (from residue last 30 amino acids ~345 to the C-terminus) required for binding of the two subunits.^{16,17}

The existence of a stable tyrosyl radical in β_2 is paradoxical, given their high reduction potential,^{6,18,19} and the relatively low reduction potential of the cellular milieu. This level of redox frustration, together with the necessity of $Y_{122}\bullet$ for enzymatic activity, implies structural and/or chemical mechanisms to protect the radical. At least four such mechanisms have been proposed for the class Ia RNR of *Escherichia coli*: (1) a rigid protein fold and sequestered tyrosyl radical cofactor in the β_2 subunit;^{8,11} (2) a thermodynamically uphill RT pathway;^{20,21} (3) a tight complex formed once the radical enters the RT pathway;¹⁰ and, (4) multiple allosteric mechanisms that conformationally gate RT, which occurs only after the formation of an intact pathway.^{9,22–24} Among these potential protective mechanisms, conformational (allosteric) gating of RT within the $\alpha_2\beta_2$ subunit is the least understood.

An exemplar allosteric RT regulation mechanism in the RNR complex involves quaternary structural rearrangements, from an active $\alpha_2\beta_2$ structure to an inactive $\alpha_4\beta_4$ structure, induced by dATP binding to the activity site.^{24,25} This structural interconversion is proposed to regulate activity to maintain balanced deoxynucleotide pools.²⁶ In addition, more subtle and dynamic interactions in the formation of the $\alpha_2\beta_2$ complex competent for RT are coupled to substrate and effector identities giving rise to the rate-determining step during turnover.²² Both mechanisms of allosteric RT regulation require inter-subunit communication and therefore necessarily involve the subunit interface. Whereas these dynamic interactions are manifested kinetically, they are poorly understood due to their transient nature and, as noted above, the lack of an atomic structure of the $\alpha_2\beta_2$ complex.

Numerous residues in α_2 and β_2 , proposed to be at the subunit interface based on an $\alpha_2\beta_2$ docking model,⁹ have been identified that mediate $\alpha_2\beta_2$ binding, RT, and attendant NDP reductase activity. In addition to the disordered carboxylate-rich C-terminal tail of β_2 , important in subunit interactions, two conserved anionic residues (E_{52} and E_{350}) on the β_2 protein surface, and two conserved cationic residues (R_{329} and R_{639}) on the α_2 protein surface are known to be either essential or nearly essential for activity.^{17,27,28} Due to their ionizable nature, the role of these residues in inter-subunit communication or proton transfer (PT) during RT via PCET, specifically for RT between $Y_{356}[\beta]$ and $Y_{731}[\alpha]$, is difficult to discern (Figure 1).

The residue glutamate 350 (E_{350}) in β_2 has been studied in detail in an attempt to elucidate its role in gating RT.²⁹ E_{350} is strictly conserved among the class Ia RNRs and is unresolved

(disordered, Fig. 1) in all currently available X-ray structures of β_2 . The essential nature of this residue is evident in activity assays performed on β_2 constructs with E₃₅₀ mutations (E₃₅₀X, where X = alanine [A], aspartate [D] or glutamine [Q]). These mutants yield no observable enzymatic activity (0–0.2% of wild type, WT) despite reasonable $\alpha_2\beta_2$ binding affinities.^{16,29} The use of nonsense suppression methodologies for incorporation of unnatural tyrosine analogues with perturbed redox potentials and/or pK_a 's has also offered mechanistic insight into the role of E₃₅₀. Incorporation of 3-nitrotyrosine (NO₂Y) into position Y₁₂₂ raises the radical reduction potential by >200 mV, creating a “hot oxidant” that, in the presence of α_2 , CDP and ATP, results in 0.5 equivalents of Y₃₅₆• and deoxycytidine diphosphate (dCDP, half-sites reactivity).^{21,30} Conversely, mixing of Y₁₂₂NO₂Y:E₃₅₀D β_2 with CDP/ATP loaded α_2 shows no NO₂Y₁₂₂• loss due to RT, no dNDP formation and no Y₃₅₆• formation.²⁹ Additionally, incorporation of 3,5-difluoro-tyrosine (F₂Y) at pathway residues Y₃₅₆[β] and Y₇₃₁[α] has been performed to test the role of E₃₅₀ as a potential base during oxidation at these sites during RT.²⁹ The oxidation potential of F₂Y is similar to Y ($E^{\text{el}} = -30$ mV vs. Y at pH > F₂Y pK_a)^{31,32} though it is more acidic (pK_a (F₂Y) = 6.833 in aqueous solution and 7.034 at position Y₃₅₆ in RNR; pK_a (Y) = 10). Enzymatic activity measurements at pH values above the fluorophenolic pK_a , where PT is decoupled from F₂Y oxidation, again yields no detectable RT. These results suggest that the primary role of E₃₅₀ is not as a base during either Y₃₅₆[β] or Y₇₃₁[α] oxidation, a conclusion corroborated by other studies.^{20,34,35,36} Notwithstanding, the role of E₃₅₀ is obscured by our inability to induce RT such that pathway residue oxidation “downstream” from Y₁₂₂ (Y₃₅₆[β], Y₇₃₁[α], Y₇₃₀[α], or C₄₃₉[α]) may be examined. Thus, to address the functional implications of E₃₅₀ on the RT pathway, novel tools for generating radicals downstream from Y₁₂₂ are required.

In this report we describe the utilization of a photosensitized RNR to investigate the fidelity of the RT pathway in isolation from Y₁₂₂• reduction within an E₃₅₀Q mutant β_2 , which our previous studies have shown eliminates all RNR activity.²⁹ We now show that RNR activity of E₃₅₀Q β_2 may be rescued by the photochemical generation of a tyrosyl radical at position Y₃₅₆. In this approach, a rhenium(I) 1,10-phenanthroline bromomethylpyridinyl chromophore ([Re^I]-Br) is covalently ligated to the β_2 subunit via a thioether linkage at a cysteine residue installed in place of S₃₅₅, directly adjacent to the RT pathway residue Y₃₅₆, a construct we have termed photo β_2 .^{37,38} Excitation of [Re^I] in the photo β_2 furnishes Y₃₅₆• on the RT pathway, which is chemically and kinetically competent for stimulating RNR activity.^{37,38} The coupling of radical generation to absorption of a photon from a temporally short laser pulse affords RT events and protein dynamics to be examined by time resolved spectroscopic methods that are decoupled from the rate determining conformational gating step.^{34,39} To this end, spectroscopic and kinetic analyses reveals that the E₃₅₀ residue is not involved in the requisite PCET events necessary to generate either Y₃₅₆•[β] or Y₇₃₁•[α], and that radicals generated at Y₃₅₆ are both kinetically and chemically competent for dNDP production, suggesting a role of E₃₅₀ in long range allosteric regulation of Y₁₂₂• reduction.

Methods

Materials and molecular biology, protein expression, purification, [Re^I] ligation and F₂Y synthesis methods are provided in the Supplementary Information.

K_D measurements.

K_D measurements were performed by both the competitive inhibition assay previously developed¹⁶ and a modified emission lifetime titration-based method described below. For the competitive inhibition assay, reaction mixtures contained 0.15 μM WT α_2 , 0.3 μM WT β_2 (reconstitution yield of 1.1 $Y\cdot\beta_2$), 1 mM CDP, 3 mM ATP, 50 μM TR, 1 μM TRR, 0.2 mM NADPH and 0–5 μM $E_{350}\text{Q}$ -photo β_2 in standard assay buffer (50 mM HEPES, 1 mM EDTA, 15 mM MgSO_4 , 5% glycerol adjusted to pH 7.6 by 6 M NaOH). The reaction was monitored continuously at 340 nm for consumption of NADPH over one minute. The data were fit to,

$$[E_{350}\text{Q}]_{\text{bound}} = \frac{[E_{350}\text{Q}]_{\text{max}} \cdot [E_{350}\text{Q}]_{\text{free}}}{K_D + [E_{350}\text{Q}]_{\text{free}}} \quad (1)$$

where $E_{350}\text{Q}$ is shorthand notation for $E_{350}\text{Q}$ -photo β_2 , $[E_{350}\text{Q}]_{\text{bound}}$ is the concentration of the $\alpha_2/E_{350}\text{Q}$ -photo β_2 complex, $[E_{350}\text{Q}]_{\text{max}}$ is the concentration of the $\alpha_2/E_{350}\text{Q}$ -photo β_2 complex at maximal $[E_{350}\text{Q}]_{\text{free}}$ and K_D is the dissociation constant for $E_{350}\text{Q}$ -photo β_2 with α_2 . This analysis assumes that the $\alpha_2\beta_2$ complex concentration at different concentrations of $E_{350}\text{Q}$ -photo β_2 inhibitor scales with activity.

The K_D of the $E_{350}\text{Q}$ -photo $\beta_2:\alpha_2$ complex was independently evaluated by an emission lifetime-based assay of the $E_{350}\text{Q}$ -photo β_2 in the presence of 0–10 μM $Y_{731}\text{F}$ α_2 (where F indicates phenylalanine) with 1 mM CDP and 3 mM ATP in assay buffer. Due to sequestration of the $[\text{Re}^I]$ moiety within the $\alpha_2\beta_2$ interface upon complexation, the emission lifetime increases as a function of $\alpha_2\beta_2$ complex concentration,³⁸ allowing the emission lifetime to report on $\alpha_2:\beta_2$ association/dissociation. Here we assume that $[E_{350}\text{Q}]_{\text{bound}}$ of eq (1) is analogous to the observed emission lifetime (τ_{obs}), $[E_{350}\text{Q}]_{\text{max}}$ is analogous to the maximum lifetime observed under completely complexed $E_{350}\text{Q}$ -photo β_2 (τ_{max}), the $\alpha_2\beta_2$ complex population is modulated identically by concentration changes in free α_2 ($[Y_{731}\text{F}]_{\text{free}}$) or β_2 ($[E_{350}\text{Q}]_{\text{free}}$), and the data can be scaled by the free $E_{350}\text{Q}$ -photo β_2 emission lifetime (τ_0) as,

$$\frac{\tau_{\text{obs}}}{\tau_0} = 1 + \frac{\left(\frac{\tau_{\text{max}}}{\tau_0}\right) \cdot [Y_{731}\text{F}]_{\text{free}}}{K_D + [Y_{731}\text{F}]_{\text{free}}} \quad (2)$$

The factor of 1 represents a scaling factor, as this analysis is a relative measurement (unitless), whereas the analysis in eq (1) is absolute. This analysis assumes $Y_{731}\text{F}$ binds with an identical K_D as WT α_2 .

Photochemical turnover assay.

Photochemical turnover experiments were performed similarly to previous reports using a radiometric quantitation of $[^3\text{H}]$ -dCDP production.^{37,40} Briefly, assays were performed in a 1 cm quartz cuvette at room temperature under illumination from a 150 W Xe arc lamp

equipped with a 320 nm long pass optical filter. 300 μ L samples of 10 μ M α_2 , 20 μ M photo β_2 , 0.2 mM 5- $^{[3]}\text{H}$ -cytidine diphosphate ($^{[3]}\text{H}$ -CDP, 32,000 cpm/nmol), 3 mM ATP and 10 mM $\text{Ru}(\text{NH}_3)_6\text{Cl}_3$ were used for all experiments using either standard assay buffer or a mixed buffer system for pH dependent measurements consisting of 50 mM HEPES and 50 mM MES with 1 mM EDTA and 15 mM MgSO_4 5% glycerol pH adjusted to 6.2 or 8.2 by 6 M NaOH. α_2 and photo β_2 yielded similar background signals for experiments performed in the absence of light; the values from these experiments were averaged and designated 0 dCDP/ α_2 , to which all subsequent measurements are referenced.

Time resolved emission.

Time resolved emission and absorption measurements were performed on a home-built optical nanosecond time resolved instrument described previously.³⁸ Samples for emission lifetime measurement contained 2 μ M photo β_2 , 5 μ M α_2 , 1 mM CDP and 3 mM ATP in standard assay buffer. The entire sample volume (550 μ L) was recirculated by a peristaltic pump through a 2 mm $\times$ 10 mm cylindrically-bored quartz cuvette during the course of the experiment. Sample excitation was achieved by the frequency tripled output of an Nd:YAG laser (355 nm, 2 mJ/pulse) and the emission was collected via a series of lenses, slits and a monochromator directed to a photomultiplier tube (PMT). Spectral resolution was determined by the spectrophotometer entrance and exit slits to be 0.25 nm and emission traces collected at 575 nm. A long pass filter ($\lambda > 375$ nm) was used to partially reject the scattered excitation pump light. Data were recorded in 50 shot intervals and in triplicate to confirm no excitation dependence of measured lifetimes. Emission traces were fit to a convoluted instrument response and mono-exponential decay function to account for both instrument rise, laser scatter signature and emission decay. Error from sample to sample was significantly larger than the error associated with lifetime fitting. Lifetimes are therefore reported as the average of three independent sample measurements and error represents one standard deviation among the independent measurements.

Charge separation rate constants (k_{CS}) were determined from, where

$$k_{\text{CS}} = \left(\frac{1}{\tau_{\text{obs}}} - \frac{1}{\tau_0} \right) \quad (3)$$

where τ_{obs} is the observed lifetime and τ_0 is a reference lifetime ($\text{E}_{350}\text{Q}:\text{Y}_{356}\text{F}$ -photo β_2 / Y_{731}F α_2). This analysis assumes all non-radiative $[\text{Re}^{\text{I}}]^*$ decay pathways remain identical between samples except for the charge separation component.

Transient absorption.

Samples were prepared with 30 μ M photo β_2 , 50 μ M α_2 , 1 mM CDP, 3 mM ATP and 10 mM $\text{Ru}(\text{NH}_3)_6\text{Cl}_2$ in either assay buffer or the identical pH dependent photochemical turnover assay buffer (50 mM HEPES/MES). The sample volume (650 μ L) was recirculated and filtered by an inline 0.22 μ m PES membrane filter to remove insoluble material generated by the reduction of the ruthenium flash quencher and enzyme oxidation.

Transient absorption (TA) lifetime and spectra measurements were performed on the same instrument with minor modifications. The probe light was generated by a Xe-arc lamp which was filtered ($\lambda > 375$ nm).

For TA lifetimes, the observation wavelength was selected by the spectrophotometer ($\lambda_{\text{ots}} = 410$ nm for Y•; 400 nm for F₂Y; 0.45 nm resolution). Transient signals were collected at 2 ns digital resolution and linearly binned to 100 ns resolution to decrease noise. 1000 shots were averaged in 100 shot collection intervals to ensure samples did not degrade during the course of the measurement. The PMT background current pre-trigger was used as I_0 for absorbance calculations where $A = -\log(I/I_0)$. The TA kinetic traces displayed are representative traces of three independent measurements fit to a single exponential function from 200 ns to 90 μ s. Lifetime error represent one standard deviation among three independently prepared samples.

For TA spectral measurements, signals were collected on a thermoelectrically cooled CCD camera with 0.45 nm resolution (as determined by slits) and wavelength binned to 2 nm resolution (~ 4 pixels/nm) from 1–1.05 μ s after excitation. Spectra were collected in a protocol controlled by shutters and delay generators and analyzed according to Eq 4 to eliminate signatures induced by the probe light,

$$\Delta A = -\log\left(\frac{I_{\text{pump on: probe on}}}{I_{\text{pump off: probe off}}} - \frac{I_{\text{pump off: probe on}}}{I_{\text{pump off: probe off}}}\right) \quad (4)$$

Spectra displayed represent the average of three independent samples composed of 10×100 shot spectra. Sub-spectra were examined as a function of exposure and showed very little signal drift (<20 – 30%) over the course of the 1000 exposures.

Results

The role of the E₃₅₀ residue on RT was probed by constructing a photo β_2 with an E₃₅₀Q mutation that was introduced by site directed mutagenesis of the corresponding photo β_2 (see Supplemental Information). The protein(s) were expressed, purified to homogeneity and labeled with the [Re^I] unit according to previously developed protocols.^{37,38} We chose the relatively conservative E₃₅₀Q mutation to preclude sidechain ionization, while maintaining similar H-bonding and steric character.

K_D for E₃₅₀Q-photo β_2 : α_2 Interaction.

The K_D for α/β interaction in WT $\alpha_2\beta_2$ RNR is relatively weak ($K_D = 0.2$ μ M) and hence we examined the K_D for the α_2 :E₃₅₀Q-photo β_2 complex under the requisite conditions of our experimental studies. The photo β_2 construct provides a convenient mechanism for measuring K_D since the emission of the [Re^I]^{*} excited state is significantly perturbed by the local environment. Figure 2 shows the normalized emission lifetime of the E₃₅₀Q-photo β_2 as a function of Y₇₃₁F α_2 concentration. Y₇₃₁F α_2 was chosen to prevent potential [Re^I]^{*} quenching by oxidation of Y₇₃₁. This mutation has a minimal effect on the complex K_D (*vide infra*). Analysis of the effect of added Y₇₃₁F α_2 on E₃₅₀Q-photo β_2 emission lifetimes

by fitting the experimental data to eq (2) yielded a K_D of 1.1(2) μM . This measurement was verified by the traditional competitive inhibition assay described by eq (1), which yielded a similar K_D of 1.5(3) μM (Figure 2 inset). The K_D of the E_{350}Q -photo β_2 : α_2 interaction strength is intermediary between the two previously reported values for E_{350}A β_2 (0.48 μM from Sjöberg et al.¹⁷ and 2.2 μM from Stubbe et al.²⁹) and slightly elevated from the standard photo β_2 (0.7 μM ³⁸). The measured K_D informs the concentrations used in all subsequent experiments such that >90% $\alpha_2\beta_2$ stoichiometry is maintained.

Photochemical Turnover of E_{350}Q -photo β_2 .

The E_{350}Q -photo β_2 was tested for photochemical dNDP production activity in single turnover experiments.^{37,40} In this assay, turnover is limited by the inherent reducing equivalents of the α_2 subunit, which contains four cysteines per monomer that can be oxidized to disulfides during NDP reduction, corresponding to a theoretical maximum single turnover of 4 dNDP/ α_2 .⁴¹ In practice, the maximal turnover observed is <4 dCDP/ α_2 ; ~1.8 dCDP/ α_2 is typically observed for non-photochemical experiments (wt β_2 with $\text{Ru}(\text{NH}_3)_6\text{Cl}_3$ flash quencher) and 0.1–0.6 dCDP/ α_2 when the single turnover experiment is driven by light under the photochemical experimental conditions where $\text{Ru}(\text{NH}_3)_6\text{Cl}_3$ is employed as a “flash quench” reagent.^{38,42} Figure 3 shows that the photochemical turnover performance for four photo β_2 variants: photo β_2 , Y_{356}F -photo β_2 , E_{350}Q -photo β_2 , and $\text{E}_{350}\text{Q}:\text{Y}_{356}\text{F}$ -photo β_2 in complex with both WT and Y_{731}F α_2 is similar to previous photo β_2 experiments.^{38,42} These mutants were chosen to examine the role of Y_{356} (vs. the Y_{356}F variant), Y_{731} (vs. the Y_{731}F) and E_{350} (vs. E_{350}Q). The photo β_2 in complex with WT α_2 produces ~0.2 dCDP/ α_2 , corresponding to ~10% of WT β_2 turnover in the presence of flash quencher, whereas no turnover is observed in the presence of Y_{731}F α_2 . The fractional dCDP formation of photo β_2 relative to the WT β_2 is ascribed to off pathway photochemical degradation targeting non-RT pathway associated aromatic sidechains both in α and β (*vide infra*).^{38,42,43} Photochemical turnover is not observed with either WT or Y_{731}F α_2 in complex with Y_{356}F -photo β_2 . Remarkably, by comparison, E_{350}Q -photo β_2 displayed similar dCDP formation activity relative to the standard photo β_2 . Photochemical turnover was also observed in experiments performed on $\text{E}_{350}\text{Q}:\text{Y}_{356}\text{F}$ -photo β_2 in complex with WT α_2 , in contrast to that of Y_{356}F -photo β_2 . The corresponding control experiment with Y_{731}F α_2 and $\text{E}_{350}\text{Q}:\text{Y}_{356}\text{F}$ -photo β_2 yielded no turnover. This result implies that the E_{350}Q mutation may affect Y_{731} conformation, enabling direct radical injection into α_2 via Y_{731} . To test this possibility, emission quenching experiments were undertaken on E_{350}Q -photo β_2 to acquire insight into the radical generation and transfer kinetics.

Kinetics of Radical Generation in E_{350}Q :photo β_2 s.

The roles of $\text{Y}_{356}[\beta]$ and $\text{Y}_{731}[\alpha]$ in kinetics of photochemical radical generation in E_{350}Q -photo β_2 and $\text{E}_{350}\text{Q}:\text{Y}_{356}\text{F}$ -photo β_2 were examined by emission lifetime measurements. The emission traces were well described by single exponentials (Figure S1); Table 1 lists the lifetimes and corresponding charge separation rate constants (k_{CS}). The radiative lifetime in the absence of a Y oxidation pathway (τ_0) is furnished from the $\text{E}_{350}\text{Q}:\text{Y}_{356}\text{F}$ -photo β_2 emission lifetime in complex with Y_{731}F ($\tau_{\text{obs}} = 680$ (5) ns). With this reference for T_0 , k_{CS} rates in the presence of either Y_{731} (WT α_2), Y_{356} (E_{350}Q -photo β_2) or both residues may be determined from eq (3). Consistent with the photochemical turnover assays, the significant

k_{CS} of $0.9 (1) \times 10^5 \text{ s}^{-1}$ for the $E_{350}Q:Y_{356}F\text{-photo}\beta_2$ construct in complex with WT α_2 suggests that Y_{731} quenches the $[Re^I]^*$ state in the absence of $Y_{356}[\beta]$ to drive RT and nucleotide reduction (Figure 3). Moreover, the measured k_{CS} of $1.6(2) \times 10^5 \text{ s}^{-1}$ for $E_{350}Q\text{-photo}\beta_2$ relative to $E_{350}Q:Y_{356}F\text{-photo}\beta_2$ in complex with $Y_{731}F \alpha_2$ indicates that oxidation of $Y_{356}[\beta]$ is more efficient than oxidation of $Y_{731}[\alpha]$. Finally, with both $Y_{731}[\alpha]$ and $Y_{356}[\beta]$ available for photochemical oxidation ($E_{350}Q\text{-photo}\beta_2$ with WT α_2), we observe the largest k_{CS} ($=2.2(2) \times 10^5 \text{ s}^{-1}$), consistent with the presence of two $[Re^I]^*$ quenching pathways.

Kinetics of RT in $E_{350}Q\text{-photo}\beta_2$ s.

To directly probe Y_{356} photochemical oxidation and the potential subsequent RT into α_2 via Y_{731} , transient absorption (TA) measurements were performed on both $E_{350}Q\text{-photo}\beta_2$ and $E_{350}Q:Y_{356}F\text{-photo}\beta_2$. TA spectra were recorded 1 μs after excitation of $E_{350}Q\text{-photo}\beta_2$ and $E_{350}Q:Y_{356}F\text{-photo}\beta_2$ in complex with $Y_{731}F \alpha_2$ in the presence of 10 mM $\text{Ru}(\text{NH}_3)_6\text{Cl}_2$ flash quencher in standard assay buffer. The TA spectrum of $E_{350}Q:Y_{356}F\text{-photo}\beta_2$ (green) shown in Figure 4 displays a weak and broad feature at $\sim 410 \text{ nm}$, consistent with a $Y^\bullet$ species not associated with Y_{356} . The increasing baseline from 450–500 nm is also consistent with the presence of a small amount of a $W^{+\bullet}$ species. This spectrum is essentially identical to those previously reported by us⁴⁴ when the Y_{356} residue has been mutated to F; the signal arises from off-pathway aromatic sidechain oxidation(s) that presumably deactivates the enzyme and circumvents complete single turnover competency (*vide supra*). The TA spectrum of $E_{350}Q\text{-photo}\beta_2$ generated under identical conditions contains a significantly enhanced $Y^\bullet$ feature at 410 nm and a shoulder at 388 nm. The absorption intensity of this feature is $5\times$ that of the corresponding $E_{350}Q:Y_{356}F\text{-photo}\beta_2$ spectrum, and is identical to the transient $Y_{356}^\bullet$ spectrum observed under similar conditions in the standard $\text{photo}\beta_2$.³⁷ These spectra provide direct evidence of Y_{356} oxidation in the presence of the $E_{350}Q$ mutation.

The temporal behavior of the photogenerated $Y_{356}^\bullet$ in $E_{350}Q\text{-photo}\beta_2$ complexed with either $Y_{731}F$ or WT α_2 was examined to determine the kinetic fate of the radical. Figure 5 shows the TA kinetics probed at the $Y^\bullet$ feature maxima (410 nm) over 90 μs after excitation and reveals kinetic differences in the decay constants of $Y_{356}^\bullet$ within the $E_{350}Q\text{-photo}\beta_2:\alpha_2$ complexes. The observed $Y_{356}^\bullet$ lifetime in the presence of $Y_{731}F$, where no pathway for radical decay into α_2 is present is 17.1 (8) μs , whereas the $Y_{356}^\bullet$ lifetime in the presence of WT α_2 (and substrate) is 13.7(7) μs .

Proton-Decoupled Y Analog, $Y_{356}F_2Y\text{-photo}\beta_2$.

The transient spectra in Figure 4 suggest that Y_{356} oxidation occurs by a PCET mechanism that does not depend on E_{350} as a base. To examine this more thoroughly, nonsense suppression was employed to site specifically install 3,5-difluorotyrosine (F_2Y) at position Y_{356} within the $E_{350}Q\text{-photo}\beta_2$ background (see Supplemental Information). Fluorotyrosines (F_nY s) allow $Y^\bullet$ behavior to be decoupled from PT due to the significantly lower phenolic pK_a (7.0).^{34,45} The lowered pK_a of F_2Y relative to Y allows kinetics in radical generation and propagation within a single construct to be elucidated in the presence and absence of a proton. Figure S2 shows that photochemical single-turnover at pH 6.2 and

8.2 is similar at both pHs and is also similar to standard photo β_2 . At pH 6.2, the F₂Y-OH population is predicted to be 86%, whereas at pH 8.2 the population is 6%. The characteristic transient signal of 3,5-F₂Y• ($\lambda_{\text{max}} = 398 \text{ nm}$) is observed from the E₃₅₀Q:Y₃₅₆F₂Y-photo β_2 complex at pH 8.2, whereas an additional broad background feature is observed at pH 6.2 complicating interpretation (Figure S3).

Noting that the $\alpha_2\beta_2$ complex is somewhat unstable at pH 6.2 over extended periods of time (>5 min) and the presence of tyrosinate at higher pH, we chose to examine radical dynamics under pure ET conditions by studying E₃₅₀Q:Y₃₅₆F₂Y-photo β_2 at pH 8.2, above the pK_a of F₂Y₃₅₆. The F_nY is more efficient than Y at quenching the excited state, consistent with a PT decoupled radical generation process, which is less kinetically hindered. Emission lifetimes of the Y₃₅₆F₂Y-photo β_2 and E₃₅₀Q:Y₃₅₆F₂Y-photo β_2 in complex with either Y₇₃₁F or WT α_2 indicated significant excited state quenching relative to the corresponding E₃₅₀Q:Y₃₅₆F-photo β_2 control construct at pH 8.2 (Table S1). The kinetics of F₂Y₃₅₆• decay in the presence of either WT or Y₇₃₁F at pH 8.2 were the same within the error limits of the experiment ($\tau_{\text{obs}} = 19.2$ (7) and 20 (1) μs , respectively), and therefore it is inconclusive from the TA measurements whether or not F₂Y₃₅₆• is injected into α_2 at this high pH, though photochemical turnover data supports RT into the α_2 subunit.

Discussion

Despite extensive efforts, no RT has been observed for RNR bearing an E₃₅₀X[β] (X = A, D or Q) mutation,²⁹ thus establishing, and at the same time circumventing any insight into, its essential role in enzymatic activity. Three proposed scenarios for E₃₅₀ function include (1) a proton acceptor during Y₃₅₆[β] oxidation, (2) a proton acceptor during Y₇₃₁[α] oxidation, or (3) a participant residue in allosteric signaling across the interface of the α/β complex. To probe these possibilities, we pursued a photo β_2 strategy, which permits RT to be initiated at either Y₃₅₆[β] or Y₇₃₁[α] while perturbing E₃₅₀. We chose to implement the photo β_2 strategy on the E₃₅₀Q β_2 variant because this mutation, together with [Re^I] ligation, minimally perturb the α/β interface as indicated by the binding constant of E₃₅₀Q-photo β_2 to α_2 ($K_D \sim 1.2 \mu\text{M}$, Figure 2) in conjunction with the complete inactivity of the non-photosensitized β_2 form.²⁹ The activity of the E₃₅₀Q β_2 mutant is photochemically “rescued” by the E₃₅₀Q-photo β_2 approach and thus offers a strategy to probe the role of this essential amino acid in regulating RNR activity.

Our observation of photochemical turnover in E₃₅₀Q-photo β_2 establishes that the RT pathway into and within α_2 remains intact. By flash quenching the [Re^I]* state with Ru(NH₃)₆Cl₃, a long lived [Re^{II}] state is generated, thus facilitating TA measurements by circumventing charge recombination. Examining the TA spectra of E₃₅₀Q-photo β_2 and E₃₅₀:Y₃₅₆F-photo β_2 (Figure 4), it is clear that Y₃₅₆ is indeed photochemically oxidized to a tyrosyl radical despite the non-ionizable nature of the Q₃₅₀ sidechain. The photochemically generated Y₃₅₆• is sensitive to the RT pathway in α_2 and appears to equilibrate onto the pathway to ultimately activate the CDP substrate for reduction. The decay kinetics of Y₃₅₆• were shown by TA (Figure 5) to be distinct in the presence of the intact RT pathway compared to a blocked pathway in α_2 (Y₇₃₁F), a hallmark of radical injection that we have characterized previously.^{37,42} These results directly show that E₃₅₀ is not a requisite base for

Y₃₅₆ oxidation. Similar results were obtained for E₃₅₀Q:Y₃₅₆F₂Y-photoβ₂, which is also capable of photochemical F₂Y oxidation above and below the fluorophenolic pK_a (Figure S3). The similar TA amplitude of the F₂Y₃₅₆• feature at pH 6.2 and 8.2, despite a broad baseline feature at pH 6.2, indicates that the [Re^{II}] state in E₃₅₀Q:Y₃₅₆F₂Y-photoβ₂ can oxidize F₂Y₃₅₆ by both PCET (pH 6.2) and ET (pH 8.2) mechanisms. This construct was also competent for photochemical turnover at both pHs (Figure S2), demonstrating that the F₂Y₃₅₆• can be reduced by Y₇₃₁[α], thus entering the RT pathway in α.

That E₃₅₀Q:Y₃₅₆F-photoβ₂, where the essential pathway residue Y₃₅₆ is rendered redox inert, is also competent for phototurnover, however, leaves the role of Y₃₅₆ unclear from this data alone. For this mutant, it is possible that Y₃₅₆• may not be the primary product of [Re^{II}] reduction, and that Y₇₃₁[α] is accessible for oxidation in E₃₅₀Q:Y₃₅₆F-photoβ₂. We have previously observed conformational flexibility of Y₇₃₁[α] in X-ray structures of 3-aminotyrosine (NH₂Y) substituted α₂ (Y₇₃₀ NH₂Y), where Y₇₃₁ occupies both the canonical π-π stacked conformation with Y₇₃₀ (or in this case NH₂Y₇₃₀), termed the “Y-Y dyad,” as well as a “flipped out” conformation where the Y₇₃₁ sidechain moves towards the α/β interface.⁴⁶ In addition, our previous studies have also shown an R₄₁₁A mutation in α accentuates the flipped out conformation of Y₇₃₁, established by pulsed electron double resonance (PELDOR) and electron nuclear double resonance (ENDOR) spectroscopies,⁴⁷ presumably by disrupting the H-bonding network involved in stabilizing the Y-Y dyad. The flipping out of Y₇₃₁ and its potential for interaction with the [Re^I]* was evidenced clearly by both photochemical turnover of the Y₃₅₆F-photoβ₂ in the presence of R₄₁₁A α₂, but not R₄₁₁A:Y₇₃₁F α₂, and by emission lifetime measurements that demonstrated [Re^I]* quenching by Y₇₃₁ in the absence of Y₃₅₆ (Y₃₅₆F-photoβ₂).³⁹ These studies establish precedence for functionally relevant conformational dynamics at Y₇₃₁ that may enable direct photochemical oxidation of Y₇₃₁ in the E₃₅₀Q:Y₃₅₆F-photoβ₂/α₂ complex. This hypothesis is consistent with the aforementioned photochemical turnover of E₃₅₀Q:Y₃₅₆F-photoβ₂ in complex with WT, but not Y₇₃₁F α₂. Additional support is provided by [Re^I]* lifetime measurements of E₃₅₀Q:Y₃₅₆F-photoβ₂ (Table 1) where the observed lifetime in the presence of WT α₂ is significantly decreased relative to Y₇₃₁F α₂, demonstrating that direct photochemical oxidation of Y₇₃₁ occurs. Both R₄₁₁A and E₃₅₀Q mutations convert a charged sidechain to a neutral one that appears to be influential on the flipped out/π-π stacked equilibrium.

Considering the initial amplitude of the Y• feature in Figure 4, we conclude that Y₃₅₆• is the dominant photoproduct of the oxidatively quenched [Re^I]* state. This conclusion is further supported by the similar amplitudes of the Y• feature in the TA kinetics experiments in Figure 5 and moreover indicates that this species is capable of transferring into α₂ on timescales consistent with turnover. The kinetic evidence for rapid Y₃₅₆• transfer into α₂, as well as the photochemical turnover activity of the E₃₅₀Q:Y₃₅₆F-photoβ₂ construct is incompatible with the role of E₃₅₀[β] as a requisite base for Y oxidation (scenarios (1) and (2) described above). These results, together with the complete inactivity of E₃₅₀ mutations, suggest E₃₅₀ is a key residue involved in the requisite conformational change(s) that gates RT.

As with E₃₅₀, we²⁸ and others⁴⁸ have identified other residues at the $\alpha_2\beta_2$ interface in the *Escherichia coli* class Ia RNR that are conserved among this class and have dramatic effects on activity. Mutagenesis studies targeting E₅₂[β], R₃₂₉M and R₆₃₉[α] have yielded similar results to those of E₃₅₀[β], where relatively conservative mutations have dramatically diminished activity, while only slightly diminishing subunit affinity ($K_D \sim 0.6\text{--}5\times$ WT).²⁸ E₅₂ in particular has been informative since, unlike E₃₅₀, this residue is resolved in a number of X-ray structures of β_2 providing structural insight into the role of E₅₂, where E₃₅₀ is lacking.^{8,11} Mutations E₅₂X, where X = A, D, Q, result in complete enzyme inactivation, analogous to E₃₅₀. Interestingly, the E₅₂Q mutant displayed a K_D of 0.12 μM , slightly tighter than the WT β_2 value of 0.2 μM , motivating further study. Studies of a double mutant, where Y₁₂₂ was replaced with 2,3,5-F₃Y (E₅₂Q:Y₁₂₂F₃Y, which significantly increases the Y• reduction potential), resulted in partial decoupling of RT from conformational gating when mixed with substrate and effector loaded α_2 , ultimately generating Y₃₅₆• and dCDP with half-sites reactivity, trapping an active $\alpha_2\beta_2$ structure long enough for pull-down studies and negative stain EM.²⁸

Based on this work, a mechanism has been proposed by which E₅₂ may convey allosteric information from α_2 displayed at the subunit interface. This posit was based partially on two observations: (1) E₅₂ displays multiple conformations within the crystal structure(s) currently available including “out” towards the subunit interface, “in” towards the β_2 subunit, and an “intermediate” conformation, (2) the “in” conformation is connected via an H-bonding network to H₁₁₈, a ligand to Fe^I of the Fe₂^{III}-O/Y₁₂₂• cluster (Figures 1 and S4).²⁸ Fe^I bears the water molecule thought to provide the proton during Y₁₂₂• reduction. Thus this model involves an interaction of E₅₂[β] with α_2 that may change the stability of the “in” conformation, breaking the H-bonding chain and enabling the Fe^IH₂O unit to approach Y₁₂₂•, such that PT can be achieved. We speculate that an analogous mechanism for RT control by E₃₅₀ may be operant. Interestingly, in this study it is evident that this long range allosteric communication does not affect the fidelity of the RT pathway between Y₃₅₆ and the NDP substrate, indicating a well-defined allosteric regulation network that specifically targets Y₁₂₂• reduction. Furthermore, we note the parity of the two acidic residues in β and the two basic residues in α essential for conformational gating of *Escherichia coli* class Ia RNR. We are currently exploring the potential correlation of these residues in conveying allosteric information across the α/β subunit interface of RNR.

Regulation of intra- and inter-protein function by sole electron transfer (ET) is challenging owing to the weak distance and pathway dependence arising from electron tunneling rates relative to the general size of proteins and separation of ET cofactors. Thus inter-protein ET can involve highly heterogeneous protein-protein interfaces,⁴⁹ and intra-protein ET can explore multiple pathways.⁵⁰ In the case of RNR, it appears that coupling ET to PT (i.e. PCET) provides a unique mechanism to kinetically regulate RT, such that the essential Y₁₂₂• does not become reduced by equilibrating with Y₃₅₆ at the protein surface until a *bona fide* RT pathway is achieved within the $\alpha_2\beta_2$ complex. Thus, ionizable residues that control conformational changes are vital to regulating function, though they may not directly be involved in PCET. Such appears to be the case for E₃₅₀. While RNR may represent an extreme example of activity regulation due to its diverse substrate scope, self-regulation, and long RT distance, the ubiquity of PCET in biological catalysis suggests that similar

mechanisms of charge transfer control involving modulation of PT rather than ET coordinates may be widespread.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

RNR	ribonucleotide reductase
WT	wild type
PCET	proton-coupled electron transfer
RT	radical transfer
PT	proton transfer
ET	electron transfer
NDP	nucleoside diphosphates
dNDP	deoxynucleoside diphosphates
NTP	nucleoside triphosphates
dNTP	deoxynucleoside triphosphates
NO₂Y	3-nitrotyrosine
F₂Y	3,5-difluorotyrosine
F₃Y	2,3,5-trifluorotyrosine
[Re^I]	rhenium(I) 1,10-phenanthroline methylpyridine (β_2 ligated)
CDP	cytidine diphosphate
ATP	adenosine triphosphate

REFERENCES

- (1). Stubbe J and van der Donk W Ribonucleotide reductases: radical enzymes with suicidal tendencies. *Chem. Biol* 1995 2, 793–801. [PubMed: 8807812]
- (2). Jordan A and Reichard P Ribonucleotide reductases. *Annu. Rev. Biochem* 1998, 67, 71–98. [PubMed: 9759483]
- (3). Stubbe J and Ackles D On the mechanism of ribonucleoside diphosphate reductase from *Escherichia coli*. Evidence for 3'-C-H bond cleavage. *J. Biol. Chem* 1980, 255, 8027–8030. [PubMed: 6997288]

- (4). Licht S, Gerfen GJ, and Stubbe J Thiyl radicals in ribonucleotide reductase. *Science* 1996, 271, 477–481. [PubMed: 8560260]
- (5). Licht S and Stubbe J Mechanistic investigations of ribonucleotide reductases. *Comp. Nat. Prod. Chem* 1999, 5, 163–203.
- (6). Stubbe J and van der Donk W Protein radicals in enzyme catalysis. *Chem. Rev* 1998, 98, 705–762. [PubMed: 11848913]
- (7). Reichard P From RNA to DNA, why so many ribonucleotide reductases? *Science* 1993, 260, 1773–1777. [PubMed: 8511586]
- (8). Nordlund P and Eklund H Structure and function of the *Escherichia coli* ribonucleotide reductase protein R2. *J. Mol. Biol* 1993, 232, 123–164. [PubMed: 8331655]
- (9). Uhlin U and Eklund H Structure of ribonucleotide reductase protein R1. *Nature* 1994, 370, 533–539. [PubMed: 8052308]
- (10). Minnihan EC, Ando N, Brignole EJ, Olshansky L, Chittuluru J, Asturias FJ, Drennan CL, Nocera DG, and Stubbe J Generation of a stable, aminotyrosyl radical-induced $\alpha\beta 2$ complex of *Escherichia coli* class Ia ribonucleotide reductase. *Proc. Natl. Acad. Sci. U. S. A* 2013, 110, 3835–3840. [PubMed: 23431160]
- (11). Nordlund P, Sjöberg BM, and Eklund H Three-dimensional structure of the free radical protein of ribonucleotide reductase. *Nature* 1990, 345, 593–598. [PubMed: 2190093]
- (12). Minnihan EC, Nocera DG, and Stubbe J Reversible, long-range radical transfer in *E. coli* class Ia ribonucleotide reductase. *Acc. Chem. Res* 2013, 46, 2524–2535. [PubMed: 23730940]
- (13). Stubbe J, Nocera DG, Yee CS, and Chang MCY Radical initiation in the class I ribonucleotide reductase: Long-range proton-coupled electron transfer? *Chem. Rev* 2003, 103, 2167–2202. [PubMed: 12797828]
- (14). Wörsdörfer B; Conner DA; Yokoyama K; Livada J; Seyedsayamdost M; Jiang W; Silakov A; Stubbe J; Bollinger JM, Jr.; Krebs CJ *Am. Chem. Soc* 2013, 135, 8585–8593.
- (15). Baldwin J; Krebs C; Ley BA; Edmondson DE; Huynh BH; Bollinger JM, Jr. *J. Am. Chem. Soc* 2000, 122, 12195–12206.
- (16). Climent I, Sjöberg B-M, and Huang CY Carboxyl-terminal peptides as probes for *Escherichia coli* ribonucleotide reductase subunit interaction: Kinetic analysis of inhibition studies. *Biochemistry* 1991, 30, 5164–5171. [PubMed: 2036382]
- (17). Climent I, Sjöberg B-M, and Huang CY Site-Directed Mutagenesis and Deletion of the Carboxyl Terminus of *Escherichia coli* Ribonucleotide Reductase Protein R2. Effects on Catalytic Activity and Subunit Interaction. *Biochemistry* 1992, 31, 4801–4807. [PubMed: 1591241]
- (18). Berry BW, Martínez-Rivera MC, and Tommos C Reversible voltammograms and a Pourbaix diagram for a protein tyrosine radical. *Proc. Natl. Acad. Sci. U. S. A* 2012, 109, 9739–9743. [PubMed: 22675121]
- (19). DeFelippis MR, Murthy CP, Faraggi M, and Klapper MH Pulse radiolytic measurement of redox potentials: The tyrosine and tryptophan radicals. *Biochemistry* 1989, 28, 4847–4853. [PubMed: 2765513]
- (20). Ravichandran KR, Taguchi AT, Wei Y, Tommos C, Nocera DG, and Stubbe JA >200 meV Uphill Thermodynamic landscape for radical transport in *Escherichia coli* ribonucleotide reductase determined using fluorotyrosine-substituted enzymes. *J. Am. Chem. Soc* 2016, 138, 13706–13716. [PubMed: 28068088]
- (21). Yokoyama K, Smith AA, Corzilius B, Griffin RG, and Stubbe J Equilibration of tyrosyl radicals (Y356 $\cdot$, Y731 $\cdot$, Y730 $\cdot$) in the radical propagation pathway of the *Escherichia coli* class Ia ribonucleotide reductase. *J. Am. Chem. Soc* 2011, 133, 18420–18432. [PubMed: 21967342]
- (22). Ge J, Yu G, Ator MA, and Stubbe J Pre-Steady-State and Steady-State Kinetic Analysis of *E. coli* class I ribonucleotide reductase. *Biochemistry* 2003, 42, 10071–10083. [PubMed: 12939135]
- (23). Seyedsayamdost MR and Stubbe J Site-specific replacement of Y356 with 3,4-dihydroxyphenylalanine in the $\beta 2$ subunit of *E. coli* ribonucleotide reductase. *J. Am. Chem. Soc* 2006, 128, 2522–2523. [PubMed: 16492021]
- (24). Zimanyi CM, Chen PY-T, Kang G, Funk MA, and Drennan CL Molecular basis for allosteric specificity regulation in class Ia ribonucleotide reductase from *Escherichia coli*. *eLife* 2016, 5, e07141. [PubMed: 26754917]

- (25). Ando N, Brignole EJ, Zimanyi CM, Funk MA, Yokoyama K, Asturias FJ, Stubbe J, and Drennan CL Structural interconversions modulate activity of *Escherichia coli* ribonucleotide reductase. Proc. Natl. Acad. Sci. U. S. A 2011, 108, 21046–21051. [PubMed: 22160671]
- (26). Chen PY-T, Funk MA, Brignole EJ, and Drennan CL Disruption of an oligomeric interface prevents allosteric inhibition of *Escherichia coli* class Ia ribonucleotide reductase. J. Biol. Chem 2018, 293, 10404–10412. [PubMed: 29700111]
- (27). Hassan AQ and Stubbe J Mapping the subunit interface of ribonucleotide reductase (RNR) using photo cross-linking. Bioorg. Med. Chem. Lett 2008, 18, 5923–5925. [PubMed: 18762419]
- (28). Lin Q, Parker MJ, Taguchi AT, Ravichandran K, Kim A, Kang G, Shao J, Drennan CL, and Stubbe J Glutamate 52- β at the α/β subunit interface of *Escherichia coli* class Ia ribonucleotide reductase is essential for conformational gating of radical transfer. J. Biol. Chem 2017, 292, 9229–9239. [PubMed: 28377505]
- (29). Ravichandran K, Minnihan EC, Lin Q, Yokoyama K, Taguchi AT, Shao J, Nocera DG, and Stubbe J Glutamate 350 plays an essential role in conformational gating of long-range radical transport in *Escherichia coli* class Ia ribonucleotide reductase. Biochemistry 2017, 56, 856–868. [PubMed: 28103007]
- (30). Yokoyama K Uhlin U, and Stubbe J A hot oxidant, 3-NO₂Y122 radical, unmasks conformational gating in ribonucleotide reductase. J. Am. Chem. Soc 2010, 132, 15368–15379. [PubMed: 20929229]
- (31). Ravichandran KR, Liang L, Nocera DG, and Stubbe J Formal reduction potential of 3,5-difluorotyrosine in a structured protein: Insight into multistep radical transfer. Biochemistry 2013, 52, 8907–8915. [PubMed: 24228716]
- (32). Ravichandran KR, Zong AB, Taguchi AT, Nocera DG, Stubbe J and Tommos C Formal reduction potentials of difluorotyrosine and trifluorotyrosine protein residues: Defining the thermodynamics of multistep radical transfer. J. Am. Chem. Soc 2017, 139, 2994–3004. [PubMed: 28171730]
- (33). Seyedsayamdost MR, Reece SY, Nocera DG and Stubbe J Mono-, di-, tri-, and tetra-substituted fluorotyrosines: new probes for enzymes that use tyrosyl radicals in catalysis. J. Am. Chem. Soc 2006, 128, 1569–1579. [PubMed: 16448128]
- (34). Olshansky L, Stubbe J, and Nocera DG Charge-transfer dynamics at the α/β subunit interface of a photochemical ribonucleotide reductase. J. Am. Chem. Soc 2016, 138, 1196–1205. [PubMed: 26710997]
- (35). Nick TU, Ravichandran KR, Stubbe J, Kasanmascheff M, and Bennati M Spectroscopic evidence for a H bond network at Y₃₅₆ located at the subunit interface of active *E. coli* ribonucleotide reductase. Biochemistry 2017, 56, 3647–3656. [PubMed: 28640584]
- (36). Nick TU, Lee W, Koßmann S, Neese F, Stubbe J, and Bennati M Hydrogen bond network between amino acid radical intermediates on the proton-coupled electron transfer pathway of *E. coli* α_2 ribonucleotide reductase. J. Am. Chem. Soc 2015, 137, 289–298. [PubMed: 25516424]
- (37). Pizano AA, Lutterman DA, Holder PG, Teets TS, Stubbe J, and Nocera DG Photo-ribonucleotide reductase β_2 by selective cysteine labelling with a radical phototrigger. Proc. Natl. Acad. Sci. U. S. A 2012, 109, 39–43. [PubMed: 22171005]
- (38). Pizano AA, Olshansky L, Holder PG, Stubbe J, and Nocera DG Modulation of Y₃₅₆ Photooxidation in *E. coli* class Ia ribonucleotide reductase by Y₇₃₁ across the $\alpha_2:\beta_2$ interface. J. Am. Chem. Soc 2013, 135, 13250–13253. [PubMed: 23927429]
- (39). Greene BL, Taguchi AT, Stubbe J, and Nocera DG Conformationally dynamic radical transfer in ribonucleotide reductase. J. Am. Chem. Soc 2017, 139, 16657–16665. [PubMed: 29037038]
- (40). Steeper JR and Steuart CD A rapid assay for CDP reductase activity in mammalian cell extracts. Anal. Biochem 1970, 34, 123–130. [PubMed: 5440901]
- (41). Mao SS, Holler TP, Yu GX, Bollinger JM, Jr., Booker S, Johnston MI, and Stubbe J A model for the role of multiple cysteine residues involved in ribonucleotide reduction: amazing and still confusing. Biochemistry, 1992, 31, 9733–9743. [PubMed: 1382592]
- (42). Olshansky L, Pizano AA, Wei Y, Stubbe J, and Nocera DG Kinetics of hydrogen atom abstraction from substrate by an active site thiyl radical in ribonucleotide reductase. J. Am. Chem. Soc 2014, 136, 16210–16216. [PubMed: 25353063]

- (43). Reece SY, Seyedsayamdost MR Stubbe J, and Nocera DG Photoactive peptides for light-initiated tyrosyl radical generation and transport into ribonucleotide reductase. *J. Am. Chem. Soc* 2007, 129, 8500–8509. [PubMed: 17567129]
- (44). Olshansky L, Greene BL, Finkbeiner CL, Stubbe J, and Nocera DG Photochemical generation of a tryptophan radical within the subunit interface of ribonucleotide reductase. *Biochemistry* 2016, 55, 2718–2726.
- (45). Minnihan EC, Young DD, Schultz PG, and Stubbe J Incorporation of fluorotyrosines into ribonucleotide reductase using an evolved, polyspecific aminoacyl-tRNA synthetase. *J. Am. Chem. Soc* 2011, 133, 15942–15945. [PubMed: 21913683]
- (46). Minnihan EC, Seyedsayamdost MR, Uhlin U and Stubbe J Kinetics of radical intermediate formation and deoxynucleotide production in 3-aminotyrosine-substituted *Escherichia coli* ribonucleotide reductases. *J. Am. Chem. Soc* 2011, 133, 9430–9440. [PubMed: 21612216]
- (47). Kasanmascheff M, Lee W, Nick TU, Stubbe J, and Bennati M Radical transfer in *E. coli* ribonucleotide reductase: a NH2Y731/R411A- α mutant unmasks a new conformation of the pathway residue 731. *Chem. Sci* 2016, 7, 2170–2178. [PubMed: 29899944]
- (48). Lundin D, Torrents E, Poole AM, and Sjöberg BM RNRdb, a curated database of the universal enzyme family ribonucleotide reductase, reveals a high level of misannotation in sequence deposited to Genbank. *BMC Genomics* 2009, 10, 589–597. [PubMed: 19995434]
- (49). Nocek JM, Zhou JS, De Forest S, Priyadarshy S, Beratan DN, Onuchic JN, and Hoffman BM Theory and practice of electron transfer within protein-protein complexes: Application to the multidomain binding of cytochrome c by cytochrome c peroxidase. *Chem. Rev* 1996, 96, 2459–2489. [PubMed: 11848833]
- (50). Beratan DN, Liu C, Migliore A, Polizzi NF, Skourtis SS, Zhang P, and Zhang Y Charge transfer in dynamical biosystems, or the treachery of (static) images. *Acc. Chem. Res* 2015, 48, 474–481. [PubMed: 25307316]

Figure 1.

Radical transfer (RT) pathway of *Escherichia coli* class Ia RNR. Redox active amino acids known to be involved in PCET, formation of the $\text{Fe}_2^{\text{III}}\text{-O/Y122}\cdot$ cofactor, or conformational gating are labeled. Residues that are disordered in the X-ray crystallographic structures of β_2 are also labeled. The subject of this paper, E₃₅₀, is shown at the nexus of putative H^+ transfer (orange arrows) pathways to either Y₃₅₆[β] or Y₇₃₁[α] or a conformational gating network between substrate and effector on α_2 and Y₁₂₂•[β] (green arrow) in β_2 . Proton and electron transfer directions are indicated with arrows. Nucleoside diphosphate substrate, NDP, and (deoxy)nucleoside triphosphate allosteric effector, (d)NTP (X = H, OH), are also shown.

Figure 2.

K_D determination for $E_{350}Q$ -photo β_2 with $Y_{731}F$ α_2 . $[Re^I]^*$ emission lifetime measurements as a function of $[Y_{731}F]$ concentration (blue circles) and associated fit to experimental data based on eq (2). Error bars indicate one standard deviation among triplicate lifetime measurements. Inset shows activity inhibition K_D assay (orange circles) with associated fit based on eq (1). Error bars indicate one standard deviation from triplicate measurements.

Figure 3.

Photochemical single-turnover assays and WT standard. Photochemical samples (columns 1–4) contained 20 μ M β_2 as indicated and 10 μ M of either WT (orange) or Y₇₃₁F (blue) α_2 with 0.2 mM [³H]-CDP (32,000 cpm/nmole), 3 mM ATP and 10 mM Ru(NH₃)₆Cl₂ in standard assay buffer and illuminated for 4 min. WT β_2 standard was performed identically except no flash quencher was added.

Figure 4. Transient absorption spectrum of $E_{350}\text{Q-photo}\beta_2$ and $E_{350}\text{Q:Y}_{356}\text{F-photo}\beta_2$ following laser induced flash quenching. Samples were prepared at 30 μM photo β_2 , 50 μM Y $_{731}\text{F}$ α_2 , 1 mM CDP, 3 mM ATP and 10 mM $\text{Ru}(\text{NH}_3)_6\text{Cl}_3$ in assay buffer pH 7.6. Spectra were generated 1 μs after excitation and represent the average of 3 independent samples of 1000 laser shots.

Figure 5. $Y_{356}\bullet$ decay kinetics within the $E_{350}Q$ -photo β_2 -WT and $E_{350}Q$ -photo β_2 - $Y_{731}F$ interface probed by single wavelength TA ($\lambda_{obs} = 410$ nm). Sample conditions identical to those of Figure 3. Transients represent the average of 1000 shots on a representative sample and associated fit to a single exponential function. $Y_{731}F$ data was duplicated and offset (translucent blue) for clearer evaluation of the fit.

Table 1.[Re^I]* emission lifetimes and $\alpha_2\beta_2$ complex K_D measurements.

α_2	photo β_2	K_D (μM)	τ_{obs} (ns)	k_{CS} (10^5 s^{-1})
Y ₇₃₁ F	E ₃₅₀ Q:Y ₃₅₆ F	n.d.	680 (5)	τ_0
WT	E ₃₅₀ Q:Y ₃₅₆ F	n.d.	642 (5)	0.9 (1)
Y ₇₃₁ F	E ₃₅₀ Q	1.1 (2) ^a	615 (8)	1.6 (2)
WT	E ₃₅₀ Q	1.5 (3) ^b	593 (8)	2.2 (2)

K_D measurements determined by an emission lifetime titration (^a) and by the competitive inhibition assay (^b). Error represents the standard deviation among triplicate measurements. n.d. not determined.