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Gated Proton Release During Radical Transfer at the Subunit Interface of Ribonucleotide Reductase

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Abstract

The class Ia ribonucleotide reductase of *Escherichia coli* requires strict regulation of long-range radical transfer between two subunits, α and β , through a series of redox-active amino acids ($Y_{122}[\beta] \leftrightarrow W_{48}[\beta] \leftrightarrow Y_{356}[\beta] \leftrightarrow Y_{731}[\alpha] \leftrightarrow Y_{730}[\alpha] \leftrightarrow C_{439}[\alpha]$). Nowhere is this more precarious than at the subunit interface. Here we show that the oxidation of Y_{356} is regulated by proton release involving a specific residue, $E_{52}[\beta]$, which is proposed to order a polar channel at the subunit interface for rapid proton transfer to the bulk solvent. An $E_{52}Q$ variant is incapable of Y_{356} oxidation via the native radical transfer pathway or non-native photochemical oxidation, following photosensitization by covalent attachment of a photooxidant at position 355 $[\beta]$. Substitution of Y_{356} for various F_nY analogs in an $E_{52}Q$ -photo β_2 , where the sidechain remains deprotonated, recovered photochemical enzymatic turnover. Transient absorption and emission

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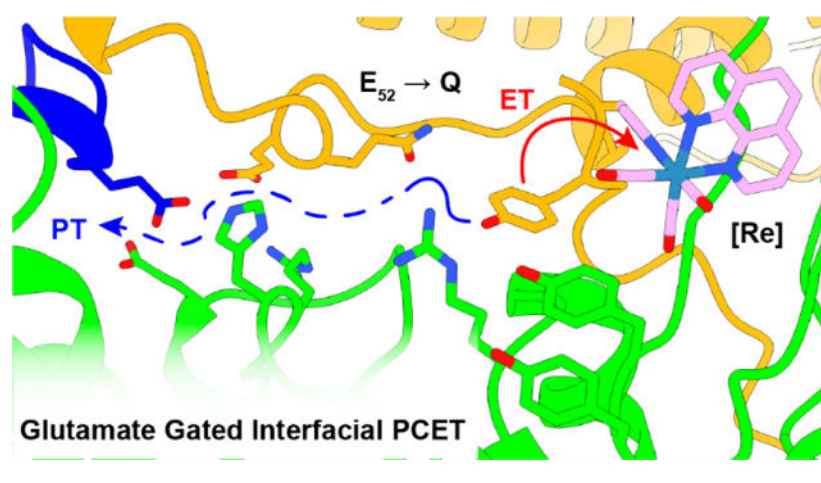
Supporting Information

PCET quenching and Radical transport model, photophysical schemes describing rate constants, schematic of laser set-up, [Re] docking model, K_d measurement of $E_{52}Q$ -photo β_2 , emission kinetic traces and fits, $E_{52}Q$ -photo β_2 vs. $E_{52}Q/Y_{356}F$ -photo β_2 transient absorption spectra. This material is available free of charge via the Internet at <http://pubs.acs.org/>

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data support the conclusion that Y₃₅₆ oxidation requires E₅₂ as a proton acceptor, suggesting its essential role in gating radical transport across the protein-protein interface.

Graphical Abstract



INTRODUCTION

Class Ia ribonucleotide reductases (RNRs) require two subunits (α_2 and β_2) for the reduction of nucleoside diphosphates (NDPs) to deoxynucleoside diphosphates (dNDPs, Figure 1).^{1,2} *E. coli* Ia RNR utilizes an $\alpha_2\beta_2$ complex for activity. Subunit α_2 controls the allosteric regulation sites that govern specificity and activity for dNDP formation³ and is the site of the reduction reaction. Subunit β_2 houses the essential diferric-tyrosyl radical (Y•) cofactor, which generates the thiyl radical in α that initiates substrate reduction (Figure 1). For the *E. coli* class Ia RNR, the distance between the stable metallo-cofactor in β_2 and the substrate in α_2 was proposed to be ~ 35 Å. This distance was based on a symmetric $\alpha_2\beta_2$ docking model of Uhlin and Eklund using the structures of each subunit⁴ and pulsed electron-electron double resonance (PELDOR) studies⁵ using a mechanism based inhibitor of RNR and using site-specifically incorporated unnatural redox active tyrosine analogs (UAA).⁶ The PELDOR studies revealed an asymmetry within the $\alpha_2\beta_2$ complex, originally suggested by studies of Ehrenberg.⁷ Efforts to obtain a structure of an “active complex” of any class I RNR remained elusive due to the weak and dynamic interactions of α_2 and β_2 .

Very recently using a double mutant of β_2 (E₅₂Q/2,3,5-F₃Y₁₂₂•) incubated with substrate GDP, specificity effector TTP, and wt- α_2 , allowed for the trapping of an active asymmetric complex of $\alpha_2\beta_2$, which was structurally characterized by cryo-EM (Figure 2A).⁸ In this complex the C-terminal tail (residues 341–375) of β was revealed for the first time in one of the two β_2 s; the second β tail remains disordered. This tail has been shown to be essential in $\alpha_2\beta_2$ subunit interactions^{9,10} and also to contain several essential residues, including Y₃₅₆ and E₃₅₀ proposed to reside at the α/β subunit interface in the proposed 35 Å radical transfer (RT) pathway (Figure 2B). Over such an extended distance, RT proceeds through distinct radical “hopping” events along a pathway of amino acids (Y₁₂₂•[β] ↔ W₄₈?[β] ↔ Y₃₅₆[β] ↔ Y₇₃₁[α] ↔ Y₇₃₀[α] ↔ C₄₃₉[α]) in a reversible and conformationally gated manner.¹¹

The thermodynamics of tyrosine oxidation necessitate that proton release be coupled to the electron transfer step at physiological pH, i.e., that radical transport occurs by proton-coupled electron transfer (PCET).^{12,13} In α_2 , the transport of the radical occurs via a colinear PCET mechanism, wherein both the proton and electron transfer between the same residues of the pathway.^{14–16} Conversely in β_2 , the distance between Y₁₂₂ and the interfacial Y₃₅₆, suggest orthogonal PCET where the electron transfers through the protein and couples to proton transfer (PT) between nearby water molecule(s) or ionizable residues within H-bonding distance.^{8,17,18} Spectroscopic investigations of the radical environment at Y₃₅₆[•] and pH dependent F_nY₁₂₂[•]/Y₃₅₆[•] (F_nYs, fluorinated tyrosines) equilibria have led us to propose that a proton must enter and exit the interface during redox cycling of this residue.^{18,19} However, on the basis of the original docking model,⁴ in which the Y₃₅₆[β] residue is buried within the protein interface, it was not obvious how a proton inventory could be maintained for orthogonal PCET. The asymmetry of the $\alpha_2\beta_2$ interaction unveiled by the cryo-EM structure reveals a path for Y₃₅₆ to release a proton that escapes the interface through a polar solvated cavity following oxidation (Figure 2C).⁸ The subunit interface presents a perilous moment during the catalytic cycle of RNR, and an uncontrolled environment here could result in radical reduction by any number of cellular reductants leading to lethal consequences. For this reason, the fidelity of RT across the subunit interface is indeed highly controlled, but the mechanism by which PT and solvation is regulated during PCET at the interface has heretofore remained poorly understood.

Mutation of a surface exposed residue at the subunit interface (E₅₂Q) in β_2 strongly inhibits RNR activity ($<10^{-3}$ the wt rate, lower limit of detection), while decreasing α/β subunit affinity by only 50% (0.18 to 0.12 μ M).^{10,20} Strikingly, substitution of Y₁₂₂[β] for 2,3,5–F₃Y₁₂₂ in the presence of E₅₂Q, strongly increased subunit affinity ($K_d < 0.4$ nM lower limit of detection) and allowed partial dNDP activity recovery in a single turnover assay (1dGDP/ α_2). The rate constants, however, for RT and the substrate turnover process remained conformationally gated and slow,^{20,21} obfuscating the function of E₅₂. Several potential roles for E₅₂ have been proposed, either conferring allosteric information from α to the Y₁₂₂[•] site in β and/or in modulating proton release/rebinding during PCET.

We have developed an approach to trigger radical transport with a photo β_2 ²² that can rescue mutant RNRs inhibited in RT and catalytic activity,²³ and that allow examination of PCET dynamics at the subunit interface.^{16,24,25} The photo β_2 methodology uses a covalently attached photooxidant (tricarboxyl(1,10-phenanthroline)-4-thiomethylpyridine, [Re]) ligated to S₃₅₅C, directly adjacent to the RT pathway residue Y₃₅₆. Excitation of the photooxidant produces the [Re^I]^{*} excited state, which can directly abstract an electron from Y₃₅₆ to generate [Re⁰] and Y₃₅₆[•]. This charge transfer process thus reports directly on radical generation, and can be interrogated by [Re^I]^{*} emission lifetimes (τ) in the presence of various Y₃₅₆X substitutions (X = F, F_nY) where F serves as the control (τ_0) that does not participate in charge transfer and F_nY are fluorotyrosines. The generation of the [Re⁰]/Y₃₅₆[•] by PCET and the subsequent radical transfer pathway are shown in in Figures 3 and S1(top). The [Re⁰]/Y₃₅₆[•] charge separated state is prone to recombination to reform the closed shell Y₃₅₆ and [Re^I] ground state. However, this recombination reaction may be avoided by oxidatively quenching the [Re^I]^{*} excited state with Ru^{III}(NH₃)₆Cl₃ to form [Re^{II}], which in turn oxidizes Y₃₅₆ to form the more stable [Re^I]/Y₃₅₆[•]. Following this sequence of events,

the Y_{356}^{\bullet} is free to propagate along the RT pathway. Radical transport may be followed by monitoring the Y^{\bullet} absorbance at 410 nm, thus reporting on the kinetics of RT within the pathway. Given the unique insight afforded by the photo β_2 method into the PCET kinetics of RT, with time resolution superseding overall conformational gating steps that obscure PCET in RNR, we employ the methodology to probe the consequences of the asymmetric RNR complex on RT at the α/β interface. The results show that Y_{356} oxidation requires H^+ release that is regulated by E_{52} and inhibited by the $E_{52}Q$ mutation, providing a rationale for the inactivity of this mutant β_2 and a gating mechanism for H^+ exchange with solvent thus enabling PCET across the interface of the $\alpha_2\beta_2$ RNR complex.

MATERIALS AND METHODS

Materials.

Luria Broth, ampicillin trisodium salt, L-arabinose, chloramphenicol, phenylmethylsulfonyl fluoride (PMSF), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), $MgSO_4$, adenosine triphosphate (ATP), deoxycytidine, and cytidine diphosphate (CDP) were purchased from Sigma-Aldrich. Isopropyl β -D-1-thiogalactopyranoside (IPTG) was purchased from GoldBio. DEAE and Q-Sepharose resins were obtained from GE Healthcare Life Sciences. Ni-NTA Sepharose resin was purchased from Qiagen. The primers for site-directed mutagenesis was obtained from Integrative DNA Technologies (IDT). BL21(DE3) *E. coli* competent cells were obtained from New England Biolabs (NEB). Tricarbonyl(1,10-phenanthroline)-(4-bromomethylpyridine)rhenium(I) hexafluorophosphate ([Re]-Br) was available from a previous study.²² [3H] CDP was purchased from ViTrax. Alkaline phosphatase (AP, calf intestine) was purchased from Roche. Thioredoxin (TR) and thioredoxin reductase (TRR) were available from a prior study.²⁰

Site directed mutagenesis.

Site directed mutagenesis was used to modify the existing pBAD photo β_2 plasmid (photo β_2 = $C_{268}S/C_{305}S/S_{355}C-\beta_2$) or $Y_{356}F$ -photo β_2 or $Y_{356}Z$ -photo β_2 to incorporate the additional $E_{52}Q$ mutation. The forward and reverse primers used were:

5'-GGAGACGTCAACCTGTTCCGGACGCC-3'

5'-GGCGTCCGGAACAGGTTGACGTCTCC-3'

and successful incorporation of the point mutation was confirmed via Sanger sequencing performed by Quintara Biosciences.

Enzymatic fluorotyrosine synthesis.

2,3,5-Trifluorotyrosine (2,3,5- F_3Y) and 3,5-difluorotyrosine (3,5- F_2Y) were synthesized enzymatically from 2,3,6-trifluorophenol or 2,6-difluorophenol via tyrosine phenol lyase (TPL) as previously described.²⁶

Protein expression and purification.

The canonical amino acid photo β_2 variants were expressed, purified, and labeled with the photosensitizer as previously reported.²²

The expression and purification of E₅₂Q/F_nY₃₅₆-photoβ₂ was accomplished using the *E. coli* BL21(DE3) expression platform transformed with both a pBAD vector encoding the E₅₂Q/F_nY₃₅₆-photoβ₂ gene, and pEVOL-aaRS-F_nY for tRNA/tRNA synthetase expression. Successful co-transformants were selected for on LB agar plates containing 100 mg/L ampicillin and 33 mg/L chloramphenicol. A single colony was inoculated into 100 mL LB medium and grown at 37 °C for 8–10 h. The starter culture was diluted into 4 × 2 L LB medium with 100 mg/L ampicillin and 33 mg/L chloramphenicol, and left to grow at 37 °C until the O.D. at 600 nm reached 0.5. pEVOL-aaRS-F_nY was then induced with 0.2% L-arabinose and the culture was supplemented with 0.7 mM F_nY. The expression of β₂ was induced by adding 0.4 mM IPTG when the O.D. attained 0.6. The cells were harvested after 5 h of over-expression, frozen in liquid nitrogen and stored at –80 °C. The growth yield was 2.5–3.0 g wet cell weight per liter media.

All protein purification steps were performed at 4 °C unless otherwise stated. The cell pellet (20 g) was thawed and re-suspended in lysis buffer (5 mL per gram of wet cell paste) containing 50 mM Tris pH 7.6, 5% glycerol, and 0.5 mM PMSF and homogenized by French Press at 13,000 psi. The lysate was supplemented with ferrous ammonium sulfate (1 mg/mL of cell lysate) and sodium ascorbate (1 mg/mL of cell lysate) dissolved in 50 mM Tris pH 7.6 and stirred for 30 min on ice prior to removing the cell debris by centrifugation at 25,000 g for 10 min. The supernatant was collected and streptomycin sulfate was added dropwise from a concentrated solution to a final concentration of 1% (w/v) and allowed to equilibrate for 30 min on ice to precipitate DNA, followed by centrifugation at 25,000 g for 10 min. The protein in the supernatant was precipitated with (NH₄)₂SO₄ (39 g per 100 mL of cell lysate) while stirring for 30 min on ice. The protein pellet was collected by centrifugation at 25,000 g for 15 min and re-dissolved in a minimal volume of lysis buffer and desalted by Sephadex G-25 column (100 mL), which was pre-equilibrated with the lysis buffer. The protein fractions were collected and loaded onto a DEAE anion exchange column (80 mL) pre-equilibrated with 50 mM Tris pH 7.6, 5% glycerol, NaCl 100 mM 0.5 mM PMSF pH 7.6, which will be referred to as buffer A hereafter, washed with 10 column volumes of buffer A and eluted with a 300 mL × 300 mL linear gradient of NaCl (100–500 mM). The fractions with absorption at 410 nm were collected and diluted three-fold with lysis buffer and loaded onto Q-Sepharose anion exchange (50 mL) column pre-equilibrated in buffer A, washed with 10 column volumes of buffer A and eluted with a 200 mL × 200 mL linear gradient of NaCl (150–500 mM) in buffer A.

Labeling E₅₂Q/F_nY₃₅₆-photoβ₂ with photooxidant.

Labeling was performed as previously described with minor modifications.²² Briefly, E₅₂Q/F_nY₃₅₆-photoβ₂ was treated 10 mM DTT and 20 mM hydroxyurea for 30 min to reduce any potential disulfide bonds as well as the endogenous Y₁₂₂•, and then separated from the reductants over a G-25 column. The labeling of the β₂ with the [Re] photooxidant was performed in 50 mM HEPES and 5% glycerol, pH 7.6. Five equivalents of [Re-Br] (50 mM in DMF) was slowly added to the protein solution with stirring. The solution was incubated at 4 °C with gentle shaking for 2 h. The protein solution was centrifuged at 25,000 rpm for 10 min to remove any precipitant and purified with G-25 column. The [Re]-labeled photoβ₂ was used for all photochemical experiments.

K_d determination.

K_d measurements were performed by the competitive inhibition assay previously developed.¹⁰ In this assay, reaction mixtures contained 0.15 μM wt α_2 , 0.3 μM wt β_2 (reconstitution yield of 1.1 $\text{Y}\cdot/\beta_2$), 1 mM CDP, 3 mM ATP, 100 μM TR, 1 μM TRR, 0.2 mM NADPH and 0–10 μM $E_{52}\text{Q}$ -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 1 min. The data were fit to:

$$[E_{52}Q]_{\text{bound}} = \frac{[E_{52}Q]_{\text{max}} \cdot [E_{52}Q]_{\text{free}}}{K_d + [E_{52}Q]_{\text{free}}} \quad (1)$$

where $E_{52}\text{Q}$ is shorthand notation for $E_{52}\text{Q}$ -photo β_2 , $[E_{52}\text{Q}]_{\text{bound}}$ is the concentration of the $E_{52}\text{Q}$ -photo β_2 : α_2 complex, $[E_{52}\text{Q}]_{\text{max}}$ is the concentration of the $E_{52}\text{Q}$ -photo β_2 : α_2 complex at maximal $[E_{52}\text{Q}]_{\text{free}}$, and K_d is the dissociation constant for $E_{52}\text{Q}$ -photo β_2 with α_2 . This analysis assumes that the $\alpha_2\beta_2$ complex concentration at different concentrations of $E_{52}\text{Q}$ -photo β_2 inhibitor scales with activity.

Single-turnover photochemical assay.

Photochemical turnover assays were carried out as previously described²⁷ with minor modification using 10 μM α_2 , 20 μM $E_{52}\text{Q}$ -photo β_2 variants, 1 mM [^3H]-CDP (31,506 cpm/nmol), 3 mM ATP, and 10 mM $\text{Ru}(\text{NH}_3)_6\text{Cl}_3$ in 50 mM HEPES, 15 mM MgSO_4 , 1 mM EDTA, and 5% glycerol at pH 7.6 (assay buffer). The total volume was 60 μL . The assay mixture was illuminated using a 150 W Xe arc lamp with a 320 nm long pass filter for 10 min at 25 $^\circ\text{C}$. The reaction was quenched by adding 60 μL of 2% ice-cold HClO_4 . Any precipitate was removed by centrifugation at 25,000 rpm for 5 min. The supernatant was neutralized with 0.4 M KOH, followed by centrifugation at 25,000 rpm for 5 min. 60 μL of the supernatant was supplemented with 12 nmol of deoxycytidine as a carrier and treated with 7 units of AP at 37 $^\circ\text{C}$ for 2 h. The [^3H]-dCDP was purified from unreacted CDP by the method of Steeper and Steward, and quantified by scintillation counting.²⁸ The reported error represents one standard deviation of triplicate measurements.

[Re]* Emission kinetics.

Time resolved emission and absorption measurements were performed on a home-built nanosecond time resolved instrument described previously and schematically represented in Figure S2.²² Emission lifetime measurements were performed on samples prepared identically as those prepared for steady state emission measurements, and the entire volume (550 μL) was recirculated by a peristaltic pump through a 2 mm \times 10 mm cylindrically bored quartz cuvette. Sample excitation was achieved by the frequency tripled output of an Nd:YAG laser (355 nm, 1–1.5 mJ/pulse) and the emission was collected via a series of lenses, slits and a monochromator directed to a photomultiplier tube. Spectral resolution was determined by spectrophotometer entrance and exit slits at 0.25 nm and collected at 575 nm, with a long pass filter ($\lambda > 375$ nm) to reject pump scattering. Data were recorded over 100 shots and measurements were performed in triplicate.

Charge separation rate constant, k_{CS} is determined from:

$$k_{CS} = \left(\frac{1}{\tau_{obs}} - \frac{1}{\tau_0} \right) \quad (2)$$

Here τ_{obs} is the observed lifetime for the $\alpha_2\beta_2$ pair of interest, whereas τ_0 is the reference lifetime in the absence of $Y_{356}[\beta]$ ($Y_{356}F$) and $Y_{731}[\alpha]$ ($Y_{731}F$). The photophysical schemes that describe τ_0 , τ_{obs} and k_{CS} are presented in Figure S1.

Transient absorption spectroscopy.

Transient absorption spectra were measured essentially as previously described²³ with 50 μM α_2 , 20 μM $E_{52}Q$ -photo β_2 variants, 1 mM CDP, 3 mM ATP, and 10 mM $\text{Ru}(\text{NH}_3)_6\text{Cl}_3$ in assay buffer. The solution was circulated with a peristaltic pump equipped with an in-line 0.22 μM syringe filter. The spectra were collected on a CCD camera from 1 μs after excitation. The pump and probe exposures were controlled by series of shutters and delay generators and the spectra were calculated by $-\log[(\text{pump on}:\text{probe on})/(\text{pump off}:\text{probe off}) - (\text{pump off}:\text{probe on})/(\text{pump off}:\text{probe off})]$. The data for each individual sample were collected and averaged over 100 laser shots and inspected for consistency, and 10 such collections per sample were averaged to produce a single TA trace. Spectra reported represent the average of three such experiments on the same photo β_2 : α_2 complex.

RESULTS AND DISCUSSION

The $E_{52}Q$ -photo β_2 was generated by site-directed mutagenesis of the corresponding photo β_2 , followed by reduction of the endogenous Y_{122}^\bullet and covalent ligation with [Re] at $S_{355}C$, directly adjacent to Y_{356} as previously described.²² Figures 4 and S3 show the [Re] complex modelled in the cryo-EM structure; the [Re] complex resides within a pocket at the interface and is situated on the opposite side of Y_{356} relative to the E_{52} -flanked channel for H^+ release. Binding studies reveal that the [Re] modification does perturb subunit interactions mildly (K_d of 1.06 (7) μM , Figure S4), consistent with all other [Re] labeled photo β_2 variants.^{16,22–24,29} The K_d for the wt subunit interactions is 0.2 μM . The perturbation in K_d is not surprising given the size and location of the [Re] group on the photo β_2 . Notwithstanding, the [Re] modification does not significantly perturb activity of the enzyme (vide infra). The asymmetry of the “active-trapped” structure (Figure 2A) and the partially disordered α'/β' interaction including the disordered β' -tail (residues 341 to 375) and partially disordered N-terminal cone domain of α' (Figure 2A, blue/red subunits) may explain why the [Re]-complex recapitulates many of the defining features of radical transfer at the subunit interface identified by orthogonal methods. For example, the conformational dynamics of Y_{731} in α_2 , observed initially by PELDOR spectroscopy in wt and $R_{411}A$ α_2 with the 3-aminotyrosine radical trap in place of Y_{731} ,³⁰ have also been clearly resolved by the photo β_2 emission kinetic and flash-quenched transient absorption experiments.¹⁶ Based on this study and others focused on the role of E_{350} - β at the subunit interface²³ and the use of $F_nY_{356}S$ - β to understand the role of proton transfer in its oxidation²⁹ as well as the distal location of [Re] to $E_{52}(Q)$, we believe that the $E_{52}Q$ -photo β_2 reports on the interactions between Y_{356} and E_{52} with fidelity.

Using this photo β_2 construct, three types of experiments have been used to assess the role of E₅₂ in the radical transfer process: (1) activity assays with E₅₂Q/3,5-F₂Y₃₅₆-photo β_2 and E₅₂Q/Y₃₅₆-photo β_2 (2) emission quenching decay kinetics [Re^I]* (Figure S1) and (3) transient absorption spectroscopic experiments with E₅₂Q/F_nY₃₅₆-photo β_2 (n = 2,3) to detect the F_nY₃₅₆[•] generation and radical transport (Figure 3).

Activity.

The flash quench technique with Ru(NH₃)₆Cl₃ was used to examine dCDP formation with E₅₂Q-photo β_2 and E₅₂Q/Y₃₅₆F₂Y-photo β_2 (Figure 5). With Y₃₅₆, no statistically significant dCDP production is observed after 10 min of illumination with respect to non-illuminated control. Thus, as with the wt- β_2 (that has a Y₁₂₂[•]), E₅₂ is essential for catalysis with photo- β_2 where Y₁₂₂ is bypassed. This inactivity suggests that either no radical is formed at Y₃₅₆ photochemically, or that the photogenerated radical is not competent for RT and/or nucleotide reduction. This behavior is distinct on mutants (E₃₅₀D, E₃₅₀N) from analogously conserved interfacial residue, E₃₅₀(β), for which E₃₅₀Q or E₃₅₀D mutations are also completely inactive with α_2/β_2 /substrate and effector. These same mutations in the photo β_2 construct, however, showed substantial photochemical recovery of activity.^{23,31}

Our previous pH-dependent activity studies using F_nY₃₅₆ analogs,^{32,33} synthesized by native protein ligation methods, and wt- β_2 ³¹ have shown that dNDP activity is maintained when F₂Y is in the protonated or deprotonated state. Thus radical transfer can occur across the subunit interface by a PCET mechanism below the pK_a of the F_nY and by ET above its pK_a. We thus prepared F₂Y₃₅₆-photo- β_2 , which our previous studies have shown has a pK_a of 7.0,²⁹ and examined its activity as well. The results of Figure 5 reveal that E₅₂Q-photo- β_2 under light irradiation can make dCDP when the tyrosine is deprotonated and thus can support ET-mediated radical transfer.

Emission Quenching.

To determine whether Y₃₅₆ can be photochemically oxidized by the [Re] photooxidant in the presence of the E₅₂Q mutation, we performed [Re^I]* emission quenching experiments on the E₅₂Q-photo β_2 in complex with either wt or Y₇₃₁F α_2 . Table 1 lists the emission quenching results for the E₅₂Q-photo β_2 systems and their respective controls (Figure S5 shows representative decay traces from which kinetics were extracted). The quenching of [Re^I]* by Y₃₅₆ oxidation leads to the formation of a transient [Re⁰]-Y₃₅₆[•], occurring with a rate constant $k_{CS} = 3.3 \times 10^5 \text{ s}^{-1}$ (Entry 2), reflecting efficient charge separation. Oxidation of Y₃₅₆ in E₅₂Q-photo β_2 is significantly retarded as reflected by a $k_{CS} = \sim 0.5 \times 10^5 \text{ s}^{-1}$ (Entries 3 and 4) using the control E₅₂Q/Y₃₅₆F-photo β_2 to determine τ_0 (Entry 5), where Y₃₅₆ has been replaced with the redox inert F. Furthermore, the quenching of [Re^I]* cannot bypass Y₃₅₆ as evidenced by the similarity of quenching lifetimes for E₅₂Q/Y₃₅₆F-photo β_2 paired with Y₇₃₁- α_2 and wt- α_2 (Entries 5 and 6, respectively). To provide further insight into the direct photooxidation of Y₃₅₆ in an E₅₂Q-photo β_2 : α_2 complex, we employed flash-quench transient absorption spectroscopy, which is sensitive to the long-lived [Re^I]-Y₃₅₆[•] state resulting from oxidative quenching of [Re^I]*. No additional absorption is observed in the characteristic Y[•] absorption region relative to the control E₅₂Q/Y₃₅₆F-photo β_2 (Figure S6). These data show Y₃₅₆ oxidation to be inhibited by the E₅₂Q mutation.

We have previously leveraged fluorinated tyrosine analogs (F_nY s, $n = 1-3$) as mechanistic probes that depress the fluorophenolic pK_a sufficiently such that F_nY oxidation occurs through ET rather than PCET at pHs above the fluorophenolic pK_a .^{29,33} Generation of a 3,5- F_2Y_{356} and 2,3,5- F_3Y_{356} substituted $E_{52}Q$ -photo β_2 ($E_{52}Q/Y_{356}F_2Y$ -photo β_2 and $E_{52}Q/Y_{356}F_3Y$ -photo β_2 , respectively) was accomplished by amber codon suppression and these variants were used to interrogate whether radical generation could be enhanced by decoupling it from proton transfer.^{24,34} Whereas 3,5- F_2Y_{356} ($pK_a = 7.0$) is partially deprotonated at pH 7.6, it is fully deprotonated at pH = 8.2; the more acidic F_3Y_{356} ($pK_a = 6.2$) is fully deprotonated at pH = 7.6.²⁹ For $E_{52}Q/3,5-F_2Y_{356}$ -photo β_2 , the emission quenching rates (Entries 7–10) are enhanced with regard to the $E_{52}Q/Y_{356}$ -photo β_2 control (Entry 5). Moreover, the quenching rate of $E_{52}Q/3,5-F_2Y_{356}$ -photo β_2 increases with 3,5- F_2Y_{356} deprotonation (Entries 7 vs 9 and Entries 8 vs 10). At pH 8.2, where the 3,5- F_2Y_{356} is completely deprotonated, the $k_{CS} = 2.5 \times 10^5 \text{ s}^{-1}$ (Entry 8) is nearly equivalent to that of $E_{52}Q/2,3,5-F_3Y_{356}$ -photo β_2 (Entry 12) at pH = 7.6. Hence, the charge separation kinetics of mutants where the proton is absent approaches that of the photo β_2 where E_{52} is not mutated (Entry 2, $k_{CS} = 3.3 (1) \times 10^5 \text{ s}^{-1}$), consistent with proton decoupling and effective radical generation and injection within the $E_{52}Q$ background. Finally, we note that in comparing the $[Re^I]^*$ emission kinetics of $E_{52}Q/Y_{356}F_nY$ -photo β_2 s in complex with wt α_2 containing an intact radical transport pathway, the rate enhancement is more pronounced (Entries 8 vs 7, 10 vs 8 and 12 vs 11), suggesting that radicals are injected into the RT pathway in α on a timescale competitive with $[Re^I]^*$ decay (i.e. RT is competitive with PCET quenching of $[Re^I]^*$ shown in Figure 3).

Transient Absorption.

Radical generation can be directly observed by transient absorption (TA) spectroscopy following flash quenching. Figure 6 compares the TA spectrum obtained for the single mutant $E_{52}Q$ -photo $\beta_2:Y_{731}F$ - α_2 construct (Figure 6A, open black circles, pH 7.6 and green dots, pH 8.2) and double mutants $E_{52}Q/Y_{356}F_2Y$ -photo β_2 (Figure 6A, open red circles, pH 7.6 and blue dots, pH 8.2) and $E_{52}Q/Y_{356}F_3Y$ -photo β_2 (Figure 6B, black dots). As we have previously observed, when Y_{356} cannot be oxidized, the hole equivalent is diverted to tryptophan residues, akin to pathway arguments made for ET proteins,^{35,36} and the broad feature at 525 to 550 nm associated with a deprotonated tryptophan radical is observed. This is the case for $E_{52}Q$ -photo β_2 where Y_{356} is protonated and the $E_{52}Q$ mutation appears to inhibit release of the phenolic proton. The tyrosine residue cannot be oxidized and a $W\bullet$ signal prevails relative to only a minor signal appearing for $Y\bullet$. We note that this off-pathway oxidation is likely responsible for a significant fraction of lost activity in photochemical turnover experiments.³⁷ For $E_{52}Q/Y_{356}F_2Y$ -photo β_2 at pH 7.6, a modest increase in $F_nY\bullet$ absorbance is observed with a concomitant decrease in $W\bullet$ congruent with a partially deprotonated F_2Y_{356} . However, when the tyrosine exists entirely as phenolate, which is the case for $E_{52}Q/Y_{356}F_2Y$ -photo β_2 at pH 8.2 and $E_{52}Q/Y_{356}F_3Y$ -photo β_2 ($pK_a = 6.2$) at pH 7.6, a pronounced $Y\bullet$ signal is observed upon photoexcitation (Figure 6A, blue dots and Figure 6B, black dots), constituting a >3-fold increase in $Y\bullet$ with the correlated loss in the relative $W\bullet$ intensity. We interpret these results to suggest that the putative water channel is blocked by $E_{52}Q$, thus interfering with interfacial PCET. The corresponding activity data for the 3,5- F_2Y_{356} mutant shown in Figure 5 is consistent with these PCET

kinetics results; E₅₂Q-photoβ₂ is able to turnover only when tyrosinate is present. The collective observations support a model where E₅₂ participates in the obligate proton release from Y₃₅₆ during oxidation, regulating radical transfer by PCET.

Role of E₅₂.

A model for the role of E₅₂-β is now possible based on the cryo-EM structure (Figure 2C) highlighting the subunit interface in the ordered α/β pair (green/orange Figure 2A). The α₂β₂ subunit interaction increases in affinity when radicals are trapped in the pathway. The K_d in the cryo-EM radical-trapped structure is <0.4 nM²⁰ vs 0.2 μM for wt.¹⁰ The Y₃₅₆• generated in this environment reveals that E₅₂ is >7 Å removed from its phenolic oxygen and >8 Å removed from Y₇₃₁-α, the next residue in the pathway to be oxidized. Figure 2C also reveals that charged residues line an empty cavity, as the resolution of the structure is insufficient for water detection. The E₃₂₆-α (green) and E₃₂₆-α' (blue) residues in Figure 2C provide direct access to the bulk solvent at the α/α' interface. This model is consistent with the data reported herein using photoβ₂ as well as additional perturbative experiments that show Y₇₃₁ to be flexible and Y₃₅₆ to participate in hydrogen-bonding. PELDOR experiments³⁰ and photoβ₂ experiments¹⁶ show Y₇₃₁ movement with rate constants much faster than RNR turnover. In addition, 94 GHz ¹H-ENDOR experiments with a trapped Y₃₅₆• using a 2,3,5-F₃Y₁₂₂•-β₂, revealed two equivalent H bonds to its oxygen assigned to waters and high-field 263 GHz EPR experiments revealed the largest perturbation of the gx component of the g-tensor of a tyrosyl radical reported to date. Computational modelling, as well,³⁸ suggests that E₅₂ can move relative to Y₃₅₆ to form a H-bonding pathway, which allows access of Y₃₅₆ through a water channel.

Based on the results shown here, we propose that the E₅₂Q mutation perturbs the H⁺ release from Y₃₅₆, following oxidation, indirectly through a water network, and ultimately to the bulk solvent. In all photoβ₂s, the [Re] unit does perturb the subunit interface to some extent, as evidenced by the elevated K_d , but the fidelity of PCET with respect to wt RNR is preserved. Although alternative mechanisms of H⁺ release through water channels that do not involve E₅₂ may exist in the absence of the [Re] unit, the water channel involving E₅₂ is critical as its mutation yields inactive enzyme in both photoβ₂ and wt-β₂. We also note that photoβ₂ without the E₅₂Q mutation exhibits a similar k_{CS} rate constant when the proton is decoupled from the RT pathway (i.e., $k_{CS} = 2.8(3) \times 10^5 \text{ s}^{-1}$ for E₅₂Q/2,3,5-F₃Y₃₅₆ (Entry 12 in Table 1) as compared to $k_{CS} = 3.3 \times 10^5 \text{ s}^{-1}$ for photoβ₂ (Entry 2 in Table 1). Though the conservative E₅₂Q mutation may potentially perturb water channels, its distal position relative to the RT pathway suggests otherwise. Cryo-EM structures using alternative trapping methods are in progress in an effort to reveal waters and the structure of E₅₂ itself relative to Y₃₅₆.

CONCLUSION

Direct kinetics measurements reveal that E₅₂ plays a critical role in managing the PCET of radical transport across the α:β interface of RNR. As opposed to the symmetric and buried interface predicted by the traditional docking model of the α₂β₂ complex, a recent cryo-EM structure of an active RNR α₂β₂ complex reveals an asymmetric interface in which E₅₂ is a

constituent of a critical pathway for H^+ to connect to a water network, and ultimately to the bulk solvent. The insight provided by this structure-function correlation rationalizes previously quizzical observations of interfacial residues possessing pK_a s consistent with that observed in aqueous solution and efficient PCET across the $\alpha:\beta$ interface. As we show herein, when E_{52} is mutated so as not to accommodate proton transfer, RT across the $\alpha:\beta$ interface of RNR is shut down. Perturbation of proton transfer within water clusters/channels via single amino acid sites is not unique to RNR. Cytochrome c oxidase (CcO) performs redox-coupled proton pumping to generate the proton motive force necessary for ATP synthesis.³⁹ During proton pumping in the D-channel, E_{242} (bovine heart CcO) serves to gate PT through a channel of conserved waters in a redox coupled manner.^{40,41} We suggest a similar mechanism is functional in the class Ia RNR of *E. coli* to protect the RT interface, while allowing for facile PT to the external solvent environment. Owing to the central role of RNRs in nucleic acid metabolism, therapeutics that inhibit distinct steps in the radical transport and chemistry of RNR lead to cytotoxicity, resulting in effective treatments of cancer.^{1,42–44} The studies reported herein show the fidelity of PCET in controlling RT across the $\alpha:\beta$ asymmetric interface and reveal an access point to disrupt RT, thus offering a potential new target for future drug design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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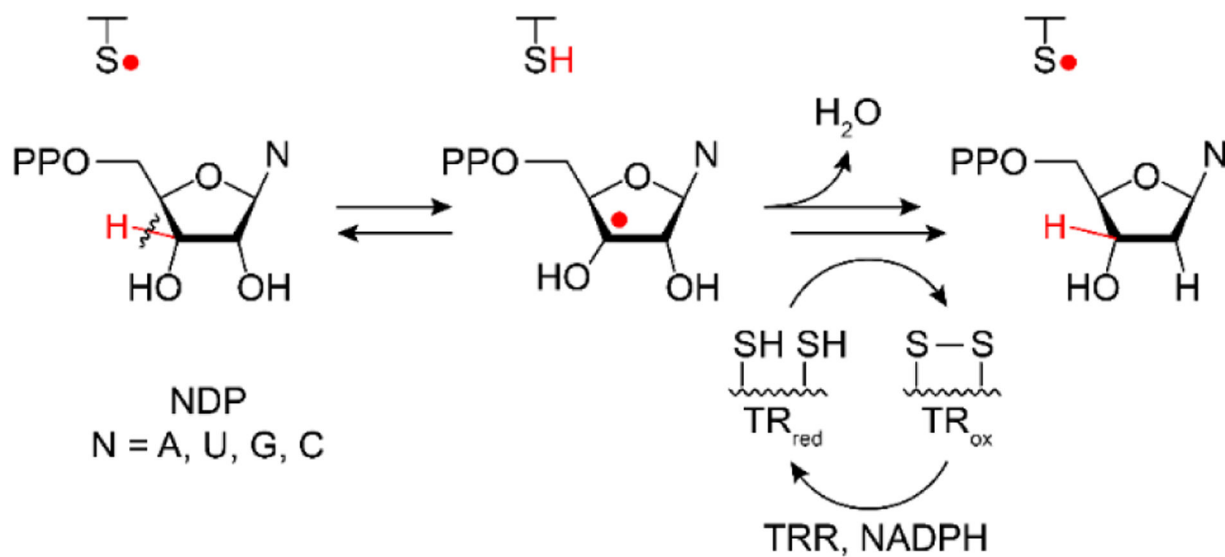


Figure 1.

Ribonucleotide reductase function. Nucleotides are “activated” for reduction by a cysteine based thyl radical mediated H-atom abstraction from the 3'-C. The substrate radical is then reduced, losing water from the 2'-C, by two cysteines in the active site that form a disulfide bond. Re-reduction of disulfide by the thioredoxin (TR), thioredoxin reductase (TRR), and NADPH system regenerates the active site for subsequent turnover. Thyl radical generation occurs through radical transfer and is the basis for class and sub-class differentiation. Adapted from reference 1.

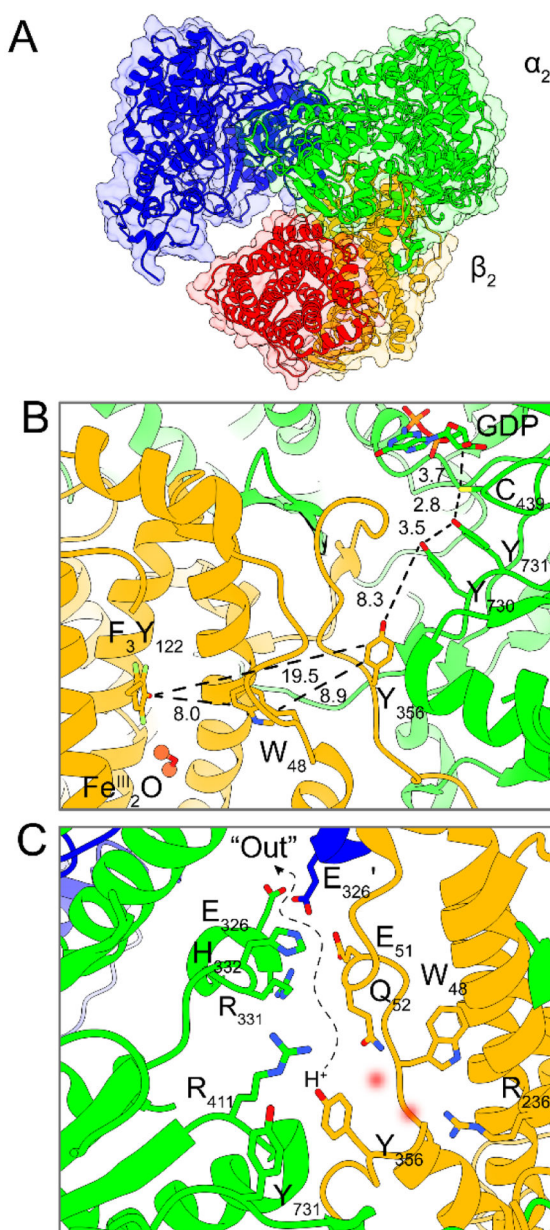


Figure 2. Cryo-EM structure of an active, asymmetric RNR $\alpha_2\beta_2$ complex (α_2 , blue and green; β_2 , orange and red). **A** Asymmetric structure of the overall complex showing an ordered α (green)/ β (orange) pair and a partially disordered α' (blue)/ β' (red) pair where the displaced α'/β' pair has already turned over, and the ordered α/β is poised for radical transfer. **B** Radical transfer pathway residues with distances in Å in the α/β pair. **C** Proposed pathway for H^+ escape following $\text{Y}_{356}[\beta]$ ($\text{Y}_{731}[\alpha]$) oxidation involving several ionizable residues and potentially ordered waters (red blurred circles) from crystallographic structures.

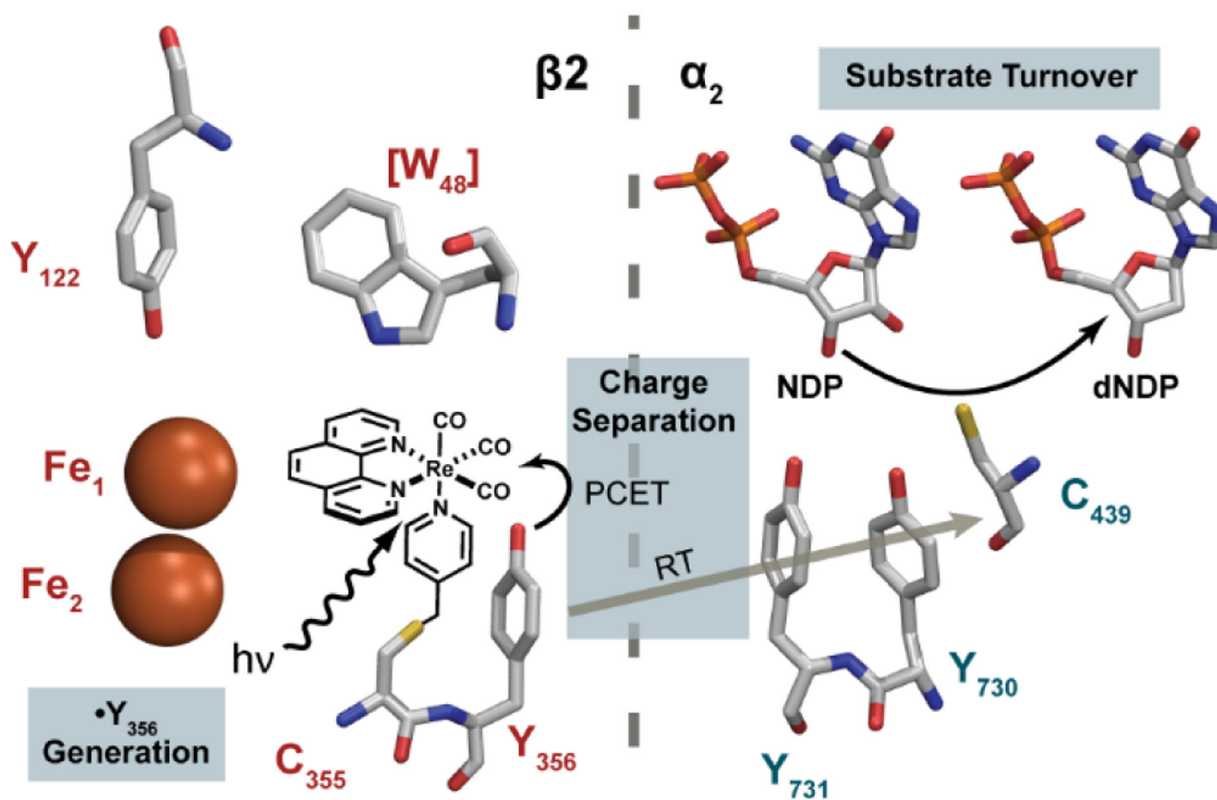


Figure 3.
Excited-state reaction pathways after excitation of $[\text{Re}^I]^*$ in photo β_2 .

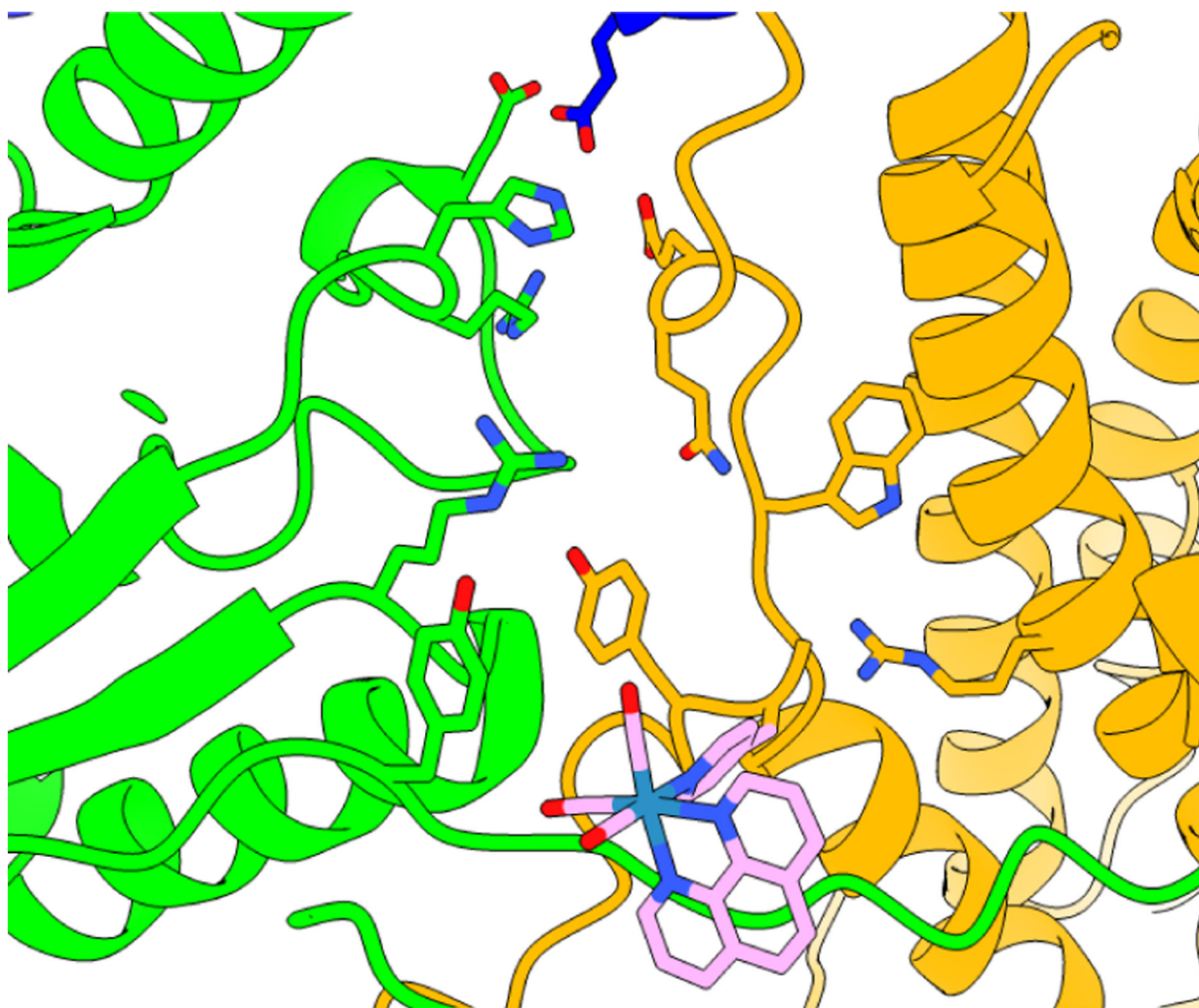


Figure 4. Docking model for the [Re] photooxidant within the $\alpha_2:\beta_2$ interface based on the crystal structure of the [Re] complex and the cryo-EM structure of the active $\alpha_2\beta_2$ *E. coli* RNR.⁸ Docking and structural refinement were performed by moving the [Re] unit so as to minimize steric contact of the chromophore and protein sidechains as much as possible, yet steric clashes do exist. This docking model is not intended to be an authentic representation of the actual structure of the complex, but it does provide a general perspective on the location of the S₃₅₅C labeling site relative to the E₅₂(Q) residue. The [Re] chromophore resides on the opposite side of Y₃₅₆ relative to the proposed polar channel of H⁺ release.

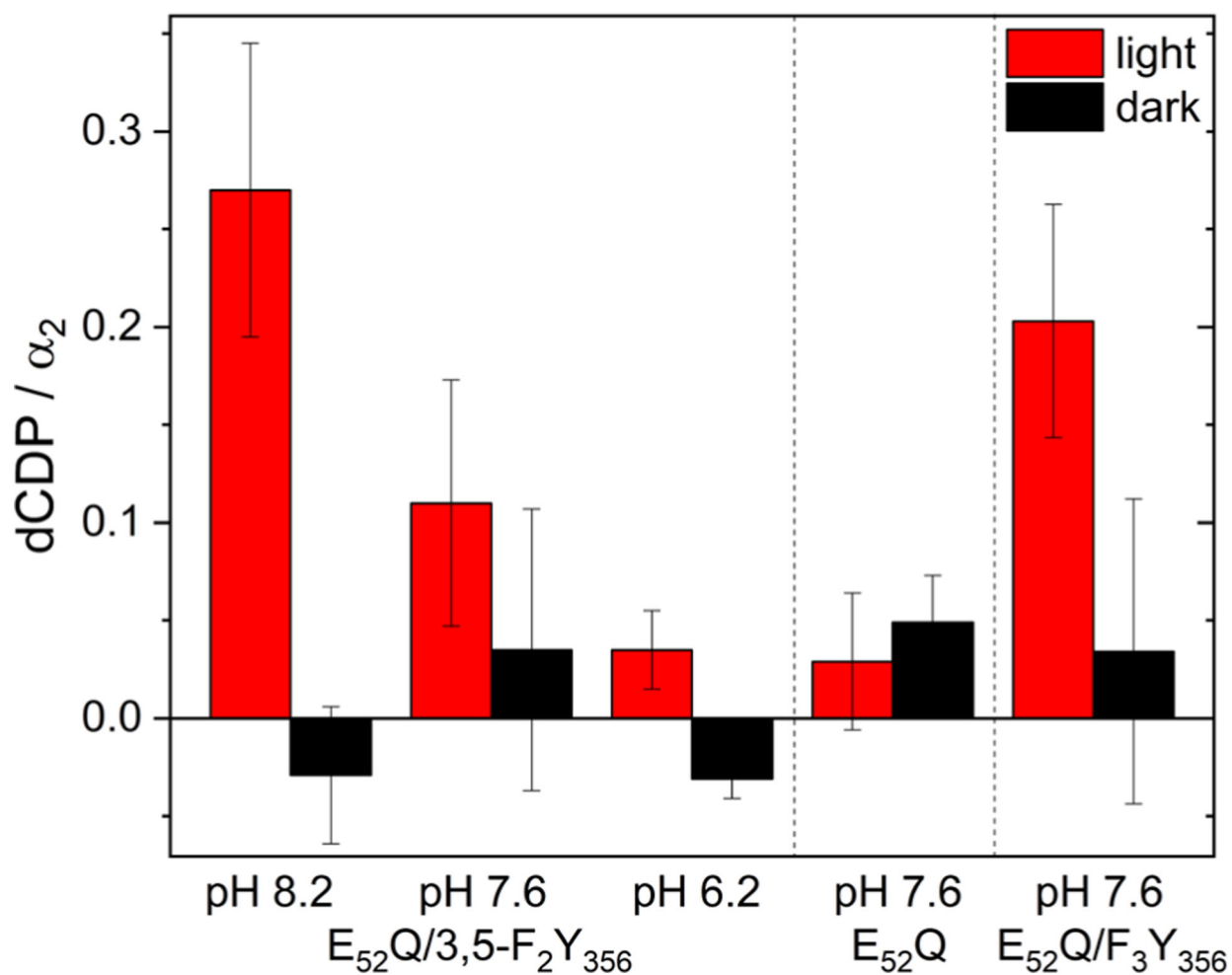
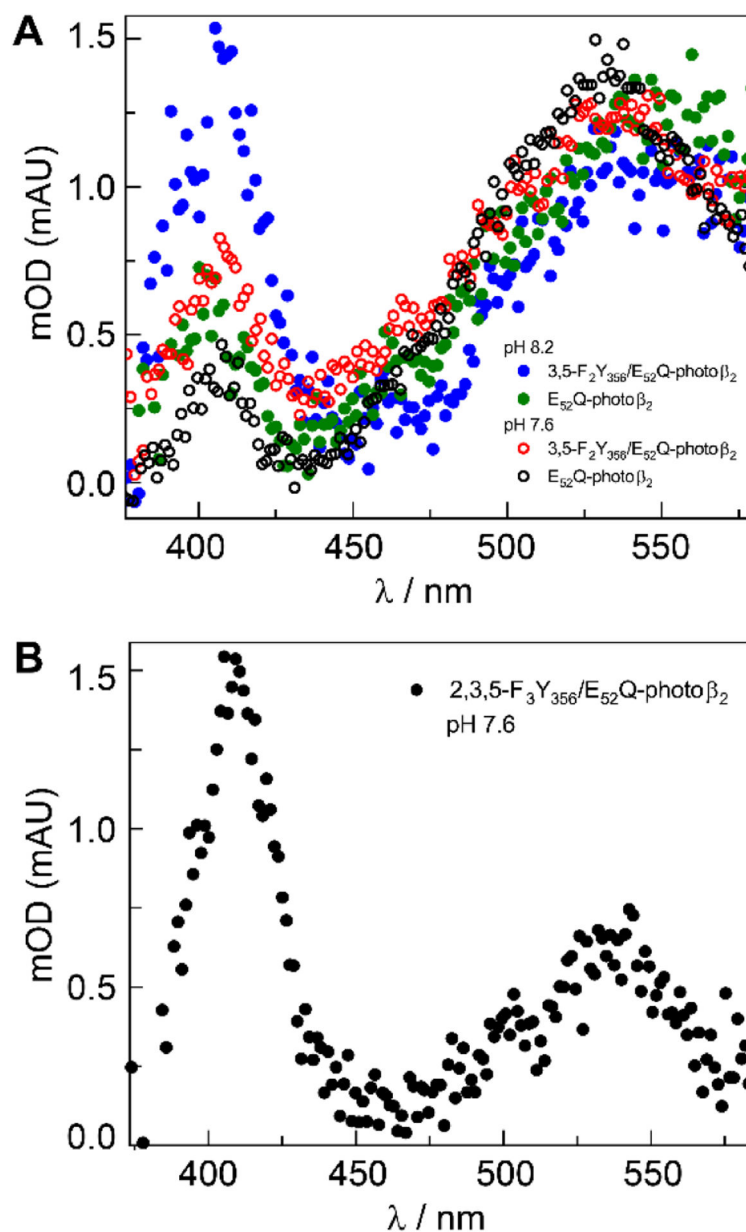


Figure 5. Single turnover photochemical assays of the $E_{52}Q$ -photo β_2 : α_2 and $E_{52}Q/Y_{356}F_2Y$ -photo β_2 : α_2 complex with 10 μ M α_2 , 10 μ M photo β_2 , 0.2 mM [3 H]-CDP substrate, 3 mM ATP effector, and 10 mM $Ru(NH_3)_6Cl_3$ in assay buffer with (red) and without (black) 10 min exposure to light ($\lambda > 320$ nm). Error bars represent one standard deviation among triplicate measurements.

**Figure 6.**

TA spectra of **A** E₅₂Q-photo β_2 :Y₇₃₁F- α_2 (○, pH 7.6; ● pH 8.2) and E₅₂Q/Y₃₅₆F₂Y-photo β_2 :Y₇₃₁F- α_2 complex (○, pH 7.6; ● pH 8.2) and **B** E₅₂Q/Y₃₅₆F₃Y-photo β_2 :Y₇₃₁F- α_2 complex at pH = 7.6. All spectra were collected at 2 μ s delay from the excitation pulse. The peak at $\lambda_{\max} \sim 540$ nm is that of W• and $\lambda_{\max} \sim 410$ nm is that of Y•.

Table 1.

Emission lifetime data (τ_{obs}) for various photo β_2 : α_2 combinations and calculated k_{CS} rates of $F_n Y_{356}$ oxidation relative to Y_{356} of $E_{52}Q$ -photo β_2 in complex with either wt or $Y_{731}F$ - α_2 .

Entry	photo β_2	α_2	pH	τ_{obs} (ns) ^a	k_{CS} (10^5 s^{-1}) ^d
1	$Y_{356}F$	$Y_{731}F$	7.6	730 (10) ^{b,c}	k_0 3.3 (1)
2	Y_{356}	$Y_{731}F$	7.6	590 (20) ^c	
3	$E_{52}Q$	$Y_{731}F$	7.6	617(5)	0.6(3)
4	$E_{52}Q$	wt	7.6	622(2)	0.5(3)
5	$E_{52}Q/Y_{356}F$	$Y_{731}F$	7.6	642(6)	k_0
6	$E_{52}Q/Y_{356}F$	wt	7.6	632(8)	n.a. ^d
7	$E_{52}Q/3,5-F_2 Y_{356}$	$Y_{731}F$	8.2	590(1)	1.4(2)
8	$E_{52}Q/3,5-F_2 Y_{356}$	wt	8.2	553(2)	2.5(3)
9	$E_{52}Q/3,5-F_2 Y_{356}$	$Y_{731}F$	7.6	596(4)	1.2(4)
10	$E_{52}Q/3,5-F_2 Y_{356}$	wt	7.6	572(5)	1.9(4)
11	$E_{52}Q/2,3,5-F_3 Y_{356}$	$Y_{731}F$	7.6	602(1)	1.0(2)
12	$E_{52}Q/2,3,5-F_3 Y_{356}$	wt	7.6	543(3)	2.8(3)

^aThe rate laws and accompanying photophysical schemes that describe τ_{obs} and k_{CS} for the various photo β_2 systems are given in Figure S1.

^bUsed as τ_0 in the calculation of k_{CS} per Eq. 2.

^cValues obtained from reference 27.

^dNot applicable.