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Glutamate 350 plays an essential role in conformational gating of long range radical transport in *E. coli* class Ia ribonucleotide reductase

Kanchana Ravichandran^{†,&}, Ellen C. Minnihan^{†,O}, Qinghui Lin[§], Kenichi Yokoyama^{†,∇}, Alexander T. Taguchi[†], Jimin Shao[§], Daniel J. Nocera^{⊥,*}, and JoAnne Stubbe^{†,‡,*}

[†]Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, United States

[‡]Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, United States

[§]Department of Pathology and Pathophysiology, Key Laboratory of Disease Proteomics of Zhejiang Province, Research Center for Air Pollution and Health, Zhejiang University School of Medicine, Hangzhou 310058, China

[⊥]Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, MA 02138, United States

Abstract

E. coli Ia ribonucleotide reductase is composed of two subunits that form an active $\alpha\beta_2$ complex. The nucleoside diphosphate substrates (NDP) are reduced in α_2 , 35 Å from the essential diferric-tyrosyl radical ($Y_{122}^{\bullet}$) cofactor in β_2 . The $Y_{122}^{\bullet}$ mediated oxidation of C_{439} in α_2 occurs by a pathway ($Y_{122} \rightleftharpoons [W_{48}] \rightleftharpoons Y_{356}$ in β_2 to $Y_{731} \rightleftharpoons Y_{730} \rightleftharpoons C_{439}$ in 7agr;2) across the α/β interface. The absence of an $\alpha\beta_2$ structure precludes insight into the location of Y_{356} and Y_{731} at the subunit interface. The sequence proximity of the conserved E_{350} to Y_{356} in β_2 suggested its importance in catalysis and/or conformational gating. To study its function, pH rate profiles of wt- β_2/α_2 and mutants in which 3,5-difluorotyrosine (F_2Y) replaces residue 356, 731 or both are reported in the presence of E_{350} or $E_{350}X$ ($X = A, D, Q$) mutants. With E_{350} , activity is maintained

*To whom correspondence should be addressed: dnocera@fas.harvard.edu, stubbe@mit.edu.

&Current address: Moderna Therapeutics, 200 Technology Square, Cambridge, MA 02139, United States

OCurrent address: Merck Research Labs, 33 Avenue Louis Pasteur, Boston, MA 02115, United States

∇Current address: Department of Biochemistry, Duke University Medical Center, Durham, North Carolina, 27710, United States

SUPPORTING INFORMATION AVAILABLE

N_3 CDP inactivation studies; detailed SF and RFQ-EPR analysis of the $NO_2Y_{122}/Y_{356}F\text{-}\beta_2/\alpha_2$ /CDP/ATP reaction; Primers and plasmids utilized in this study (Table S1); specific activity of $E_{350}Q/F_2Y_{356}\text{-}\beta_2$ (Table S2); pK_a s of F_nY measured by different methods (Table S3); pK_a s obtained by fitting the experimental pH rate profiles of F_nY -substituted RNRs to a two-proton ionization model (Table S4); kinetic parameters for the reaction of $NO_2Y_{122}/Y_{356}F\text{-}\beta_2$ with wt- α_2 , CDP, and ATP determined by SF UV-vis spectroscopy (Table S5); time-dependent inactivation of RNR by N_3 CDP (Figure S1); inactivation of $E_{350}X\text{-}\beta_2$ by N_3 CDP (Figure S1); K_d for $\alpha_2/E_{350}X\text{-}\beta_2$ ($X = A, D$ or Q) interaction (Figure S3); the pH rate profiles of F_2Y at 356 in β_2 and 731 and 730 in α_2 on the RT pathway (Figure S4); expanded pH profiles for $E_{350}D(A)/F_2Y_{356}\text{-}\beta_2$ and $E_{350}D\text{-}\beta_2/F_2Y_{731}\text{-}\alpha_2$ as determined by the radioactive assay (Figure S5); reaction of $F_3Y_{122}/E_{350}D\text{-}\beta_2$, wt- α_2 , CDP and ATP monitored by RFQ-EPR spectroscopy (Figure S6); SF UV-vis analysis of the reaction of $NO_2Y_{122}/Y_{356}F\text{-}\beta_2$ with α_2 , CDP and ATP (Figure S7); RFQ-EPR spectra of the putative W^{+} s formed in the reaction of $NO_2Y_{122}/Y_{356}F\text{-}\beta_2$ with wt- α_2 , CDP and ATP (Figure S8); PELDOR spectrum of the reaction of $NO_2Y_{122}/Y_{356}F\text{-}\beta_2$, wt- α_2 , CDP and ATP (Figure S9); reaction of $NO_2Y_{122}/E_{350}D\text{-}\beta_2$, wt- α_2 , CDP and ATP monitored by RFQ-EPR spectroscopy (Figure S10). This material is available free of charge via the Internet at <http://pubs.acs.org>.

at the pH extremes suggesting that protonated and deprotonated states of F_2Y_{356} and F_2Y_{731} are active and that radical transport (RT) can occur across the interface by proton-coupled electron transfer at low pH or electron transfer at high pH. With $E_{350}X$ mutants, all RNRs were inactive suggesting that E_{350} could be a proton acceptor during oxidation of the interface Ys. To determine if E_{350} plays a role in conformational gating the strong oxidants, $NO_2Y_{122}\cdot$ - $\beta 2$ and $2,3,5-F_3Y_{122}\cdot$ - $\beta 2$ were reacted with $\alpha 2$ /CDP/ATP in E_{350} and $E_{350}X$ backgrounds and the reactions were monitored for pathway radicals by rapid-freeze quench EPR spectroscopy. Pathway radicals are generated only when E_{350} is present, supporting its essential role in gating the conformational change(s) that initiates RT and masking its role as a proton acceptor.

Graphical Abstract

Class I ribonucleotide reductases (RNRs) catalyze the conversion of nucleoside diphosphates (NDPs) to deoxynucleoside diphosphates (dNDPs), a process involving complex free radical chemistry.^{1–4} The *E. coli* RNR is composed of α and β subunits that form the active $\alpha 2\beta 2$ complex. The radical initiation step requires a diferric-tyrosyl radical cofactor ($Y_{122}\cdot$ in *E. coli*) in one subunit ($\beta 2$) to catalyze oxidation of cysteine 439 (C_{439}) in the active site of the second subunit ($\alpha 2$).^{5, 6} This process is reversible and occurs over a distance of 35 Å (Figure 1).^{3, 4, 7} The ability to site-specifically replace a single tyrosine (Y) with a Y analog that has an altered reduction potential^{8–12} and/or pK_a ^{13, 14} and the availability of a variety of methods to monitor the kinetics of radical intermediate formation in these mutant RNRs during turnover,⁴ provide the current framework for investigation of this long-range radical transport (RT) process. The current RT pathway involves three transient $Y\cdot$ intermediates ($Y_{356}\cdot$ - β , $Y_{731}\cdot$ - α and $Y_{730}\cdot$ - α , Figure 1) with each oxidation step involving loss of a proton and an electron by proton-coupled electron transfer (PCET).^{3, 4} Orthogonal PCET is thought to initiate RT within $\beta 2$, during which the proton and electron move from different donors to the same acceptor.¹⁵ Co-linear PCET is thought to occur within $\alpha 2$, with the electron and proton originating from the same donor, an adjacent pathway residue.¹⁶ The PCET process across the α/β subunit interface, however, has remained less clear as Y_{356} resides in the structurally disordered C-terminal tail of $\beta 2$.^{17–20} This paper is focused on our efforts to understand the function of conserved E_{350} located within this disordered region that has been proposed to: (1) act as the proton acceptor during transient oxidation of $Y_{356}\cdot$ - β and/or $Y_{731}\cdot$ - α ^{21, 22} or (2) effect protein conformational gating that triggers initiation of the RT process by reduction of $Y_{122}\cdot$. To examine the function of E_{350} , RNR mutants have been prepared with unnatural amino acids (UAA; fluorotyrosines, F_nY where $n = 2, 3$; 3-nitrotyrosine, NO_2Y) site-specifically incorporated in place of pathway residues in an E_{350} or $E_{350}X$ (where X is D, A or Q) mutant background. A variety

of studies on these mutants together support the conformational gating role for E₃₅₀ in the *E. coli* class Ia RNR.

Whereas the structures of the $\alpha 2$ and $\beta 2$ subunits of *E. coli* RNR have been determined crystallographically,^{7, 17} no atomic resolution structural data are available for the proposed active $\alpha 2\beta 2$ complex. As noted above, the last 34 amino acids of *E. coli* $\beta 2$ (341–375) are structurally disordered.^{17–20} Potential insight about the location of residues 360–375 of this tail was provided by crystallization of $\alpha 2$ with a peptide corresponding to residues 355–375 of $\beta 2$,⁷ but structural information is absent for residues 341 to 359, including Y₃₅₆ and E₃₅₀ (Figure 1). Studies by Sjöberg and coworkers were the first to demonstrate that the α/β subunit affinity in *E. coli* RNR is largely governed by the last 20 amino acids of the $\beta 2$ C terminus²³ and that the K_d for the interaction is weak ($\sim 0.2 \mu\text{M}$).^{21, 23} The authors further established the importance of Y₃₅₆ and E₃₅₀, the only two conserved residues in this region, using mutagenesis.²¹ Mutation of E₃₅₀ to A in $\beta 2$ showed that the essential diferric-Y• cofactor was generated and that the K_d for the $\alpha 2/\text{E}_{350}\text{A}-\beta 2$ interaction is $0.5 \mu\text{M}$.²¹ Activity assays revealed that the mutant had 0.5% of wt-RNR activity, an amount close to the levels of endogenous RNR that almost always co-purify with any mutant of this enzyme¹. The authors suggested that E₃₅₀ is likely involved in the RT pathway between the two subunits.²¹

Recently, we evolved a polyspecific aminoacyl tRNA synthetase (RS)-tRNA pair for the incorporation of F_nYs site-specifically in place of any pathway Y in RNR (Y₁₂₂ or Y₃₅₆ in $\beta 2$, Y₇₃₁ or Y₇₃₀ in $\alpha 2$).¹⁰ The F_nY-RNRs are all active with activities that range from 5–90% of the wt-enzyme.^{4, 20} F_nYs have also been incorporated into a 65 amino acid $\alpha 3$ -helical protein and their formal reduction potentials measured and found to range from 25 mV easier to 135 mV harder to oxidize than Y.²⁴ Solution pH titration experiments of the N and C-terminally blocked amino acids demonstrated that the F_nYs cover a range of pK_as (6.4–8.4) relative to Y (10.0).²⁵ Utilizing NO₂Y site-specifically incorporated in place of each pathway Y as a probe to assess the effect of protein environment on their pK_a, we have shown that the pK_a of each transiently oxidized Y on pathway is minimally perturbed (+ 0.4 to 1 unit) compared to the solution pK_a.¹⁴ From these data, we assume in this manuscript that the F_nYs experience a similar pK_a perturbation at each pathway position.

Our early studies with 2,3-F₂Y₃₅₆- $\beta 2$ (2,3-difluorotyrosine at position 356) generated by the expressed protein ligation¹³ (EPL) method provided the first indication that F_nYs could be mechanistically useful to investigate the role of E₃₅₀. In that study, we demonstrated that 2,3-F₂Y₃₅₆ was active in dCDP production from pH 6.5 to 9.0,¹³ although the activity relative to wt RNR was low because of two additional mutations required for the semi-synthesis. These data were interpreted to suggest that dCDP could be produced regardless of the protonation state of the phenol of 2,3-F₂Y₃₅₆, i.e. that mechanisms involving PCET or ET through residue 356 can support dCDP formation. In contrast to the EPL method, the evolved F_nY-RS/tRNA pair has provided the opportunity to site-specifically replace each Y in the RT pathway and to recover protein in much higher yields and with higher specific

¹Sjöberg and coworkers concluded that E₃₅₀A- $\beta 2$ is active based on an experiment performed with the mechanism-based inhibitor 2'-azido-2'-deoxycytidine 5'-diphosphate. A description of this experiment and the caveats are presented in the SI and in Figure S1.

activities.^{4, 20} Thus, we hypothesized that a combination of the F_nY-RNRs with the E₃₅₀ mutants (to A, D or Q) could provide insight into the latter's function.

We now report steady-state kinetic studies of 3,5-difluorinated tyrosine (F₂Y) at Y₃₅₆ (F₂Y₃₅₆-β2), F₂Y₇₃₁-α2 and F₂Y₃₅₆/F₂Y₇₃₁-α2 in the presence of E₃₅₀-β2 or E₃₅₀X-β2 (X = A, D or Q, Figures 2A–C) with the second subunit, substrate (CDP), and effector (ATP) over the pH range 6.0 to 9.0. Throughout this pH range, the proteins with E₃₅₀ are active while the same proteins with an E₃₅₀X mutation are completely inactive. At the alkaline pH extreme where F₂Y phenolates are present, the inability of the E₃₅₀ mutants to make dCDP suggests a role for E₃₅₀ other than as a proton acceptor at the α/β interface. These results led to an additional set of experiments using strong oxidants incorporated in place of Y₁₂₂ (either NO₂Y₁₂₂•¹¹ or 2,3,5-trifluorotyrosine (F₃Y) F₃Y₁₂₂•¹²) both of which rapidly generate Y₃₅₆•-β2 when reacted with α2/CDP/ATP or in the former case, a tryptophan cation radical (W₄₈•⁺) when Y₃₅₆ is replaced with a redox inert phenylalanine (Y₃₅₆F). The formation of Y₃₅₆• and dCDP at 100–300 s⁻¹ and 20–30 s⁻¹, respectively is proposed to reflect unmasking of the rate-limiting conformational gating (2–10 s⁻¹ in wt RNR)²⁶ that governs all chemistry in wt RNR. These same mutants were examined in the presence of an E₃₅₀D mutation (Figures 2D–E). In contrast to the single-point mutants, no pathway radicals were detected in the presence of a second E₃₅₀D mutation. Arguments are presented that these studies and the pH rate profile analyses support the essentiality of E₃₅₀ in the conformational regulation of RT initiation.

MATERIALS AND METHODS

Materials

All primers and plasmids utilized in this study are shown in Table S1. Site-directed mutagenesis was performed using the Stratagene QuikChange kit and all constructs were confirmed by sequencing at the MIT Biopolymers Laboratory. Wt-β2 (1.2 Y•/β2, 7000 nmol/min/mg), E₃₅₀D-β2 (~1.1 Y•/β2), E₃₅₀Q-β2 (~1.1 Y•/β2) and E₃₅₀A-β2 (1.2 Y•/β2) were isolated using the standard lab protocol for wt-β2.¹⁴ Wt-α2 (2500 nmol/min/mg) was purified following the published protocol.²⁷ Thioredoxin (TR, 40 U/mg) and thioredoxin reductase (TRR, 1400 U/mg) were purified following the standard procedures.^{28, 29} Tyrosine phenol lyase (TPL) was isolated as described.³⁰ F₂Y and F₃Y were enzymatically synthesized from 2,6-difluorophenol and 2,3,6-trifluorophenol, respectively using TPL.³¹ The expression and purification of F₂Y₇₃₁-α2 (1300 nmol/min/mg) has been previously detailed.¹⁰ Apo F₃Y₁₂₂/E₃₅₀D-β2 was expressed, purified and reconstituted following our published protocol for F₃Y₁₂₂-β2.¹² NO₂Y₁₂₂/E₃₅₀D-β2 was expressed and purified as previously reported for NO₂Y₁₂₂-β2.^{11, 14} Apo NO₂Y₁₂₂/E₃₅₀D-β2 was generated from iron-loaded NO₂Y₁₂₂/E₃₅₀D-β2 as described.¹¹ 2'-Azido-2'-deoxycytidine 5'-triphosphate (N₃CTP) was obtained from TriLink Biotechnologies. 5'-[³H] CDP was purchased from ViTrax (Placentia, CA). Roche provided the calf alkaline phosphatase (20 U/μL). Rabbit muscle myosin, NO₂Y, ATP, CDP, deoxycytidine (dC), NADPH, MES, HEPES, TAPS, ampicillin (Amp), chloramphenicol (Cm) and L-arabinose (L-ara) were obtained from Sigma Aldrich. Assay buffer consists of 50 mM HEPES pH 7.6, 15 mM MgSO₄, 1 mM EDTA unless otherwise specified. Temperature was controlled at 25 °C for all experiments

using a Lauda circulating water bath. All specific activities are reported with respect to the subunit that is limiting in concentration.

K_d for α₂/E₃₅₀X-β₂ (X = A, D or Q) interaction

Subunit affinity was determined using the competitive inhibition assay.²³ Reaction mixtures contained in a total volume of 300 μL, 0.15 μM wt-α₂, 0.3 μM wt-β₂, 1 mM CDP, 1.6 mM ATP, 50 μM TR, 1 μM TRR, 0.2 mM NADPH and increasing concentrations of E₃₅₀X-β₂ (0.1–20 μM) in assay buffer. The reaction was monitored continuously for 1 min at 340 nm for the consumption of NADPH (6200 M⁻¹cm⁻¹). The data were fit to Eq. 1:

$$[E_{350}X-\beta_2]_{\text{bound}} = \frac{[E_{350}X-\beta_2]_{\text{max}} \times [E_{350}X-\beta_2]_{\text{free}}}{K_d + [E_{350}X-\beta_2]_{\text{free}}} \quad (1)$$

where [E₃₅₀X-β₂]_{bound} is the concentration of wt-α₂/E₃₅₀X-β₂ complex, [E₃₅₀X-β₂]_{max} is the concentration of the wt-α₂/E₃₅₀X-β₂ complex at maximum amounts of [E₃₅₀X-β₂]_{free} and K_d is the dissociation constant for the interaction of wt-α₂ and E₃₅₀X-β₂. Eq. 1 assumes that the amount of wt α₂β₂ complex at different concentrations of the inhibitor scales with the activity of α₂.

Expression and purification of E₃₅₀X/F₂Y₃₅₆-β₂ (X = A, D or Q)

E₃₅₀D/F₂Y₃₅₆-β₂ and E₃₅₀Q/F₂Y₃₅₆-β₂ were expressed by co-transforming pEVOL-*F_nY-RS* and either pBAD-*nrdB*-GAC₃₅₀TAG₃₅₆ (E₃₅₀D) or pBAD-*nrdB*-CAA₃₅₀TAG₃₅₆ (E₃₅₀Q) into *E. coli* Top10 chemically competent cells. E₃₅₀A/F₂Y₃₅₆-β₂ was expressed in DH10B cells from the pEVOL-*F_nY-RS* and pTrc-*nrdB*-GCA₃₅₀TAG₃₅₆ plasmids. A 2XYT culture (5 mL) with 100 μg/mL Amp and 35 μg/mL Cm was grown to saturation and was used to inoculate 5 x 500 mL of 2XYT containing the same antibiotics at a 100-fold dilution. The cells were grown until reaching an OD₆₀₀ of 0.3, at which point F₂Y was added to the media to a final concentration of 0.5 mM. At an OD₆₀₀ of 0.5, the F_nY-RS and *nrdB* gene were induced with L-ara (0.05% w/v). The cells were grown for an additional 5 h, then harvested by centrifugation (3,000 x g, 10 min, 4 °C). Typical yields of ~2 g/L were obtained.

E₃₅₀X/F₂Y₃₅₆-β₂ was purified by DEAE and Q-sepharose anion exchange chromatography following the previously reported protocol.¹⁴ SDS-PAGE analysis of the elution fractions from the Q-Sepharose column indicated the presence of the β'₂ homodimer (where β' is the β subunit truncated at residue 355), E₃₅₀X/F₂Y₃₅₆-ββ' heterodimer and E₃₅₀X/F₂Y₃₅₆-β₂ homodimer. Fractions containing the E₃₅₀X/F₂Y₃₅₆-β₂ homodimer were pooled and concentrated using an Amicon ultrafiltration cell with a YM30 membrane. Yields of 3 mg (E₃₅₀A/F₂Y₃₅₆-β₂), 16 mg (E₃₅₀D/F₂Y₃₅₆-β₂) and 10 mg (E₃₅₀Q/F₂Y₃₅₆-β₂) pure protein containing 0.95, 0.8 and 0.7 Y•β₂, respectively were obtained per g wet cell paste.

The pH rate profile of E₃₅₀X/F₂Y₃₅₆-β₂ (X = A or D)

The pH rate profile was determined using the radioactive assay for RNR.³² Assays were performed in 50 mM MES (pH 6.0–7.0), HEPES (pH 7.0–8.0) or TAPS (pH 8.0–9.0), 15 mM MgSO₄ and 1 mM EDTA. The reaction contained in a total volume of 200 μL, 4 μM

wt- α 2, 0.8 μ M E₃₅₀A/F₂Y₃₅₆- β 2 containing 0.95 Y•/ β 2 (or E₃₅₀D/F₂Y₃₅₆- β 2 containing 0.8 Y•/ β 2), 0.5 mM 5'-[³H] CDP (10,000 cpm/nmol), 3 mM ATP, 30 μ M TR, 0.5 μ M TRR, 1 mM NADPH. The reaction was initiated by the addition of E₃₅₀X/F₂Y₃₅₆- β 2 and NADPH. Aliquots (40 μ L) were quenched in 25 μ L of 2% HClO₄, neutralized by the addition of 20 μ L of 0.5 M KOH and frozen overnight (−20 °C). Samples were thawed, centrifuged (20,000 x g, 4 °C for 20 min) to remove KClO₄ and precipitated protein and the resulting supernatant was dephosphorylated by the addition of 20 U calf alkaline phosphatase, 120 nmol carrier dC in 175 mM Tris pH 8.5, 1.5 mM EDTA. Cytidine and dC were separated as described by Steeper and Steuart.³² The pH rate profile was fit to a two-proton ionization model described by Eq. 2:

$$k_{obs} = \frac{k_{max}}{1 + 10^{(pK_1 - pH)} + 10^{(pH - pK_2)}} \quad (2)$$

where k_{obs} is the specific activity at a certain pH, k_{max} is the maximum specific activity observed, and pK_1 and pK_2 are the pK values for the two proton-ionizing events, respectively.

The pH rate profile of E₃₅₀D- β 2/F₂Y₇₃₁- α 2

The pH rate profile was determined as described above for E₃₅₀X/F₂Y₃₅₆- β 2 with minor modifications. The reaction contained in a total volume of 200 μ L: 0.8 μ M F₂Y₇₃₁- α 2, 4 μ M E₃₅₀D- β 2 (1.2 Y•/ β 2), 0.5 mM 5'-[³H] CDP (8000 cpm/nmol), 3 mM ATP, 30 μ M TR, 0.5 μ M TRR and 1 mM NADPH. The pH rate profile was analyzed using Eq. 2.

Specific activity of E₃₅₀Q/F₂Y₃₅₆- β 2/F₂Y₇₃₁- α 2 at pH 6.8 and pH 8.4

Standard spectrophotometric assays were performed to assess the activity of E₃₅₀Q/F₂Y₃₅₆- β 2 and F₂Y₇₃₁- α 2. In a total volume of 300 μ L, E₃₅₀Q/F₂Y₃₅₆- β 2 (0.7 Y•/ β 2, 0.5 μ M), F₂Y₇₃₁- α 2 (4 μ M), CDP (1 mM), ATP (3 mM), TR (30 μ M), TRR (0.5 μ M) and NADPH (0.2 mM) were combined in 50 mM MES (pH 6.8) or TAPS (pH 8.4), 15 mM MgSO₄ and 1 mM EDTA. The reaction was monitored for 1 min at 340 nm.

Reaction of F₃Y₁₂₂•/E₃₅₀D- β 2, wt- α 2, CDP and ATP monitored by RFQ-EPR spectroscopy

Rapid freeze-quench (RFQ) experiments were performed on an Update Instruments 1019 syringe ram unit and a model 715 Syringe Ram controller. Wt- α 2 (80 μ M) and ATP (6 mM) in assay buffer were mixed with F₃Y₁₂₂•/E₃₅₀D- μ 2 (0.5 F₃Y•/ β 2, 80 μ M) and CDP (2 mM) in assay buffer and aged for varying times (16–535 ms) based on the previously reported kinetics for Y₃₅₆• formation.¹² The reaction mixture was then sprayed at a drive ram velocity of 1.25–1.6 cm/s into liquid nitrogen-cooled isopentane (−140 °C). The crystals were packed into X-band EPR tubes and EPR data were acquired at 77 K on a Bruker EMX X-band spectrometer equipped with a quartz finger dewar containing liquid N₂. The following EPR parameters were used: microwave frequency 9.45 GHz, power 30 μ W, modulation amplitude 1.50 G, modulation frequency 100 kHz, time constant 5.12 ms and scan time 41.93 s.

Reaction of NO₂Y₁₂₂•/E₃₅₀D-β2 or NO₂Y₁₂₂•/Y₃₅₆F-β2, wt-α2, CDP and ATP monitored by RFQ-EPR spectroscopy

Apo NO₂Y₁₂₂/E₃₅₀D-β2 (450 μL, 500 μM) was deoxygenated on a Schlenk line and taken into the anaerobic chamber at 4 °C. The protein was treated with 5 equiv. of Fe^{II}(NH₄)₂(SO₄)₂, diluted to 90 μM in anaerobic 50 mM HEPES pH 7.6, 5% glycerol and loaded into syringe A. The syringe was sealed, taken out of the anaerobic chamber and attached to the RFQ instrument. Fe²⁺-NO₂Y₁₂₂/E₃₅₀D-β2 was then mixed with O₂ saturated 50 mM HEPES pH 7.6, 5% glycerol containing 3 mM CDP from syringe B. The mixture was aged for 0.5 s¹¹ and then mixed with 90 μM wt-α2 and 9 mM ATP contained in syringe C. Samples were aged for 16–131 ms and quenched in liquid nitrogen-cooled isopentane (–140 °C) for analysis by X-band EPR spectroscopy. An identical experiment was performed with NO₂Y₁₂₂•/Y₃₅₆F-β2 except that samples were aged from 5–118 ms (see SI for more details). The different ageing times were determined based on the previously reported kinetics for diferric-NO₂Y• formation¹¹ and Y₃₅₆•¹¹/W⁺ formation (described in the SI).

RESULTS

Site-directed mutagenesis to investigate the importance of E₃₅₀

The sequence conservation of E₃₅₀ and the mutagenesis studies of Sjöberg and coworkers²¹ suggested to us that E₃₅₀ might be involved as an orthogonal proton acceptor in the oxidation of Y₃₅₆-β at the subunit interface.⁴ This proposal indicated that mutation of E₃₅₀ to A, Q and possibly D would abolish activity due to the inability to deprotonate Y₃₅₆ during oxidation. The E₃₅₀X-β2 (X = A, Q and D) mutants were prepared, purified to homogeneity and assayed for enzyme activity. The results are summarized in Table 1. The diferric-Y• cofactor for E₃₅₀X-β2 was self-assembled by the addition of ferrous ammonium sulfate and sodium ascorbate to the cell-free lysate³³ to give 1–1.1 Y•/β2, similar to the 1.2 Y• for wt-β2 (Table 1). However, the radioactive assays for RNR revealed that E₃₅₀X-β2s possess 0.2–0.4% of the wt activity, likely arising from endogenous β2. Assays in the presence of the mechanism based inhibitor N₃CDP have often been used to corroborate the inactivity of RNRs. Results from studies with E₃₅₀X-β2 (X = A, D, Q) are described in detail in the SI and in Figures S1 and S2 and support the conclusion that E₃₅₀X-β2 is not capable of catalysis. When X = D, very low levels of activity might be present. These studies support the importance of E₃₅₀ for RNR function and suggest that it could operate as the proton acceptor for Y₃₅₆ during its oxidation.

K_d for α2/E₃₅₀X-β2 (X = A, D or Q) interaction

Previous studies of Sjöberg and coworkers have shown that the K_d for the α2/E₃₅₀A-β2 interaction is 3-fold weaker than wt α2/β2.²¹ To determine the affinity of E₃₅₀X-β2 (X = A, D or Q) for α2, we used an identical protocol in which E₃₅₀X-β2 was added as a competitive inhibitor of the wt α2/β2 interaction.²³ The results are shown in Figure S3. The data were analyzed using Eq. 1 and the resulting K_d values are shown in Table 1. The K_d for subunit interaction increases ~10-fold (1.7–2.2 μM in comparison with 0.18 μM²¹ for wt) when E₃₅₀ is mutated to A, D or Q. Since RNR activity assays are typically performed with a limiting concentration of one subunit and a 5-fold excess of the second subunit, the

concentrations of the E₃₅₀ mutants and $\alpha 2$ in all subsequent experiments have been adjusted to ensure that the majority (60–100%) of $\beta 2$ is in complex with $\alpha 2$.

Role of E₃₅₀ in proton transfer (PT) at the subunit interface

pH rate profiles of RNR with F₂Y site specifically incorporated in place of Y₃₅₆- $\beta 2$, Y₇₃₁- $\alpha 2$ or both pathway Ys—F₂Y was chosen as a probe to study the function of E₃₅₀ because of its minimal perturbation to reduction potential,^{24, 25} maintained activity when substituted at position 356 of $\beta 2$ relative to wt- $\beta 2$ ^{20, 34} and the minimal perturbation introduced by the two fluorines to RNR structure.²⁰ The two important chemical properties that govern the chemistry at Y₃₅₆- β and Y₇₃₁- α are p*K*_a and reduction potential. The p*K*_a of the *N*-acetyl 3,5-difluoro-L-tyrosinamide is 7.2.^{25, 35} The pH titration experiments performed on NO₂Y₃₅₆ or NO₂Y₇₃₁ reveal that the environment minimally perturbs the p*K*_a increasing it by +0.4 and +0.9 units, respectively relative to that measured for the *N*-acetylated 3-nitro-L-tyrosinamide in solution.¹⁴ Thus, the predicted p*K*_a of F₂Y₃₅₆ is assumed to be 7.6. While the exact reduction potential of F₂Y at the subunit interface in RNR remains unknown, square wave voltammetry studies on a model protein (α_3X where X = a buried F₂Y)^{24, 36} suggest that the fluorine substitutions lower the reduction potential relative to Y by 30 mV between pH 6.0 and 9.0. Finally, F₂Y has been successfully incorporated site-specifically in place of each of the Ys in the RT pathway and the activities of these mutants have been determined to be 65 to 90% that of the wt enzyme.^{20, 34}

The pH rate profiles of wt (purple or blue) and F₂Y incorporated in place of Y₃₅₆ in $\beta 2$ (pink) and Y₇₃₁ (green) in $\alpha 2$ were generated and are shown in Figure 3 in two different representations: specific activity as a function of pH (Figures 3A–B) and percent maximal activity vs pH (Figure 3C). The specific activity representation is important as RNR is typically assayed with respect to one subunit, α or β , using a 5-fold excess of the second subunit (β or α) to ensure maximal $\alpha 2\beta 2$ complex formation. However, these assays do not give the same specific activity for $\alpha 2$ and $\beta 2$ (purple and blue in Figures 3A and 3B, respectively).²⁶ The $\beta 2$ subunit can act catalytically to turnover multiple $\alpha 2$ s, a process dependent on the protein concentrations. Due to the weak interaction between $\alpha 2$ and $\beta 2$, the activity of $\beta 2$ in typical assay conditions is almost always higher than that of $\alpha 2$. Thus, to compare the pH rate profiles of $\beta 2$ and $\alpha 2$ we normalized the data relative to the maximum activity, providing the results shown in Figure 3C. Although the absolute specific activities of $\alpha 2$ and $\beta 2$ differ, the shape of the normalized pH rate profiles of the two subunits are superimposable (Figure 3C). Finally, we note that the concentrations of the TR/TRR reducing system were optimized from pH 6.0–8.8 and are not rate-limiting at any pH value (data not shown).

The pH rate profile of wt RNR was fit to the two-proton ionization model described by Eq. 2 providing p*K*_a values of 6.8 and >9.0. The identities of the amino acid residues associated with each p*K*_a are unknown, but they are not associated with RT or CDP reduction as the rate-limiting step in wt RNR is a protein conformational change that occurs prior to these processes. The pH rate profiles of F₂Y₃₅₆- $\beta 2$ and F₂Y₇₃₁- $\alpha 2$ are shown in Figures 3A and 3B in pink and green, respectively. The activity of F₂Y₃₅₆- $\beta 2$ is 80–95% of wt- $\beta 2$ between pH 6.0 and 7.5 whereas, that of F₂Y₇₃₁- $\alpha 2$ is 40–70% of wt- $\alpha 2$ in this pH regime. When the

data are normalized as percent maximum activity, the pH rate profiles of F₂Y₃₅₆-β2 and F₂Y₇₃₁-α2 are almost identical to the wt enzyme between 6 and 7.5. The data in both cases fit to a pK_a of 7.0 vs 6.8 for the wt enzyme. From pH 7.5 to 9.0 there is a more pronounced reduction in activity for the two mutants than in wt-RNR (Figure 3C), with pK_a values of 8.0 and 8.1, respectively compared to >9.0 for wt. Interestingly, the two F₂Y mutants at the subunit interface behave similarly and are distinct from both wt RNR and the corresponding F₂Y at position 730 (Figure S4). While a molecular basis for the pH rate profiles remains to be established, the F₂Y substitution has the desired properties to examine the function of E₃₅₀. We hypothesized that if E₃₅₀ is the proton acceptor during Y₃₅₆ or Y₇₃₁ oxidation, the double mutants E₃₅₀D/F₂Y₃₅₆-β2 or E₃₅₀D-β2/F₂Y₇₃₁-α2 (Figures 2A–B) would be inactive when F₂Y is protonated as there is no proton acceptor or a poorly situated acceptor, and they would become increasingly active when the phenol becomes deprotonated as the RT process could proceed by ET instead of PCET.

The pH rate profile of E₃₅₀X/F₂Y₃₅₆-β2/α2/CDP/ATP—Our experimental design to investigate if E₃₅₀ functions as the obligate proton acceptor for Y₃₅₆ is shown in Figure 2A. E₃₅₀X/F₂Y₃₅₆-β2 (X = A, D or Q) mutants were purified to homogeneity and the diferric-Y• cofactors were self-assembled. The A and D mutants (0.8 μM) were then incubated with α2 (4.0 μM), CDP, ATP, TR, TRR and NADPH from pH 6.0 to 9.0. The pH rate profiles of wt (purple), F₂Y₃₅₆-β2 (pink), E₃₅₀D/F₂Y₃₅₆-β2 (blue), and E₃₅₀A/F₂Y₃₅₆-β2 (green) are shown in Figure 4A. Both the protonated and deprotonated forms of 3,5-F₂Y₃₅₆ are active. Figure 4A also shows the percentage of deprotonated F₂Y₃₅₆ as a function of pH (orange dots), determined from the measured pK_a perturbation at this position. At pH 6.6, F₂Y₃₅₆ is completely protonated and the mutant maintains 100% of the wt activity, whereas at pH 8.6 ~90% of F₂Y₃₅₆ is deprotonated and the protein still has ~30% of the wt activity. Maximum activity is measured at pH 7.6, even though 50% of F₂Y is deprotonated. These data agree with our earlier interpretation of studies using 2,3-F₂Y₃₅₆-β2 generated by the EPL method¹³ and suggest that the mechanism of RT switches from PCET to ET as the pH increases.

In contrast to the wt-β2 and the F₂Y₃₅₆-β2 studies, the pH rate profiles of E₃₅₀D/F₂Y₃₅₆-β2 and E₃₅₀A/F₂Y₃₅₆-β2 reveal very low activity (0.2–0.5%) over the entire pH regime (Figure 4A, blue and green and Figure S5A), within the range observed for contaminating endogenous RNR.³⁷ The activity of E₃₅₀Q/F₂Y₃₅₆-β2 was monitored at two pH values (6.6 and 8.0, Table S2), and similar to the two mutants discussed above, extremely low levels of activity were recorded. These observations suggest that E₃₅₀ is critical for catalysis, but do not allow assessment of its role as a proton acceptor during Y₃₅₆ oxidation. This provoked additional investigation of whether it is involved in either PT from Y₇₃₁ or in the conformational gating that triggers RT.

The pH rate profile of E₃₅₀D-β2/F₂Y₇₃₁-α2/CDP/ATP—As with Y₃₅₆, the proton acceptor for Y₇₃₁ during its oxidation in RT has not been identified. If E₃₅₀ functioned in this capacity, the inability to deprotonate Y₇₃₁ would result in loss of activity for E₃₅₀D/F₂Y₃₅₆-β2. The pH rate profile from 6.0–9.0 for wt α2 (purple), the single mutant wt-β2/F₂Y₇₃₁-α2 (pink), and the double mutant E₃₅₀D-β2/F₂Y₇₃₁-α2 (blue) are shown in

Figure 4B. The pK_a of F_2Y at position 731 as noted above is predicted to be 0.9 units higher than in solution.¹⁴ Therefore, if E_{350} participates in PT with Y_{731} , $E_{350}D\text{-}\beta 2/F_2Y_{731}\text{-}\alpha 2$ would be inactive when F_2Y is protonated. Figure 4B shows an overlay between the pH rate profiles and the percentage of deprotonated F_2Y_{731} as a function of pH (orange dots). As with $F_2Y_{356}\text{-}\beta 2$, $F_2Y_{731}\text{-}\alpha 2$ maintains activity in the deprotonated and protonated states of the F_2Y phenol. For example, ~97% of F_2Y_{731} is protonated at pH 6.6 and has ~60% of the wt activity whereas ~75% of F_2Y_{731} is deprotonated at pH 8.6 and maintains an activity of ~32%. In contrast to $F_2Y_{731}\text{-}\alpha 2$, the $E_{350}D\text{-}\beta 2/F_2Y_{731}\text{-}\alpha 2$ profile shows 0.3–0.7% activity at all pH values (Figure 4B and S5C). Unfortunately, this inactivity prevents assessment of the role of E_{350} as a proton acceptor for Y_{731} .

$E_{350}Q/F_2Y_{356}\text{-}\beta 2/F_2Y_{731}\text{-}\alpha 2/CDP/ATP$ does not catalyze dCDP formation at pH 6.8 or 8.4—Another possible explanation for the results described above is that E_{350} functions as the proton acceptor for both Y_{356} and Y_{731} . We have recently demonstrated that the proton from Y_{356} is in rapid exchange with solvent.³⁸ If E_{350} shuttles the Y_{356} proton to solvent and functions in a similar capacity for the Y_{731} proton, the individual mutants, $E_{350}X/F_2Y_{356}\text{-}\beta 2$ or $E_{350}D\text{-}\beta 2/F_2Y_{731}\text{-}\alpha 2$ would be inactive; with this model, activity would require the presence of a deprotonated amino acid at both positions on pathway. To test this possibility, the activity of $E_{350}Q/F_2Y_{356}\text{-}\beta 2/F_2Y_{731}\text{-}\alpha 2$ was determined at pH 6.8 and 8.4. At pH 6.8, the amounts of protonated F_2Y would be 86% and 95% at positions 356 and 731, respectively. The amount of deprotonated 3,5- F_2Y at pH 8.4 is calculated to be 86% and 66% at positions 356 and 731, respectively. The results of these assays are reported in Table 2. The activities of the single mutants, $F_2Y_{356}\text{-}\beta 2$ or $F_2Y_{731}\text{-}\alpha 2$, were taken from the pH rate profiles shown in Figures 3A (pink) and 3B (green) and reflect the inherent differences in the activity between $\alpha 2$ and $\beta 2$. The control experiment with $F_2Y_{356}\text{-}\beta 2/F_2Y_{731}\text{-}\alpha 2$ and an intact E_{350} gave activities of 810 nmol/min/mg and 1660 nmol/min/mg at pH 6.8 and 8.4, respectively establishing that RNR can function with two protonated or deprotonated 3,5- F_2Y s on pathway. No activity was detected at either pH for the $E_{350}Q$ containing triple mutant. The combined results of our studies utilizing F_2Y and $E_{350}X$ suggest that E_{350} plays a very important role in RNR function. Unfortunately, its role in PT from Y_{356} , Y_{731} or both pathway Y s cannot be assessed due to the loss of catalytic activity when mutated. The data, based on our assumptions about the pK_a perturbation of F_nY at each position in the protein, also establish that a deprotonated F_2Y at either 356 or 731 allows RT to occur through these positions via ET. In contrast to the wt RT pathway, these analogs demonstrate that PCET is not obligatory.

Assumptions and alternative interpretation of the pH rate profiles—Our conclusions in the preceding section are based on the use of NO_2Y to detect pK_a perturbations of pathway Y s within the protein environment. For Y_{356} we have also measured the pK_a s of F_nY using $[Re]^I\text{-}\beta 2$ photo-RNRs; these numbers are 0.6 units lower than predicted from the NO_2Y titration data.³⁹ We have also recently placed each F_nY in a small α_3X helical protein ($X = F_nY$) and measured their reduction potentials and pK_a s (Table S3).^{24, 36} The pK_a s of α_3F_nY are ~0.7 units higher than the solution pK_a s of the blocked amino acids. These data together suggest that F_nY s are minimally sensitive to the protein environment and that the pH titration of NO_2Y within the $\alpha 2 \beta 2$ complex is a good

estimate of pK_a perturbation. This contrasts with the $[Re]^I$ photooxidant, a bulky substituent which still minimally perturbs the pK_a and subunit affinity (10-fold weaker than wt).

The pH rate profile results for 3,5-F₂Y, 2,3,5-F₃Y and 2,3-F₂Y summarized in Table S4 allow a comparison of pK_a proposed based on the NO₂Y titrations and pK_2 calculated from Eq 2. For each F_nY at 730 in α , the measured pK_2 agrees within ± 0.3 units with the pK_a proposed by the titration method. In contrast, pK_2 for the interface Ys (Y₃₅₆ and Y₇₃₁) varies from -0.3 to $+0.7$ units from the predicted pK_a , perhaps defining the limits of the titration method. However, 3,5-F₂Y is an excellent probe to study mechanism as it is very similar to wt- $\beta 2$ (90% of wt catalytic activity, very similar structure, and minimally perturbed reduction potential.²⁰ Even if the pK_a of 3,5-F₂Y₃₅₆ varied ± 0.5 units from that predicted by the NO₂Y titrations, the high specific activity measured (Figure 3) supports that F₂Y₃₅₆ has substantial activity even when it is completely deprotonated. This observation is the basis for our interpretation that turnover can occur via ET across the subunit interface.

Interpretation of pH rate profiles for “old school” enzymologists has been notoriously difficult. In fact, the profiles of F_nY-substituted RNRs (Figure 3, Table S4)³⁴ are “very” simple. The pK_1 values are the same at each position in the pathway, suggesting that a residue with pK_a of ~ 7 must be deprotonated for either activity or more likely, for the protein conformation to trigger RT on substrate/effector binding. On the other hand, the pK_2 values are distinct with an apparent trend in activity; lower pK_a of the F_nY phenol corresponds to loss of activity at a lower pH. Although there is no direct correlation between activity and percentage of F_nY phenolate, one interpretation of the data is that pK_2 represents deprotonation of F_nY and that the F_nY phenolates are inactive. One possible explanation for the loss of activity could be weakened subunit interaction due to the introduction of a negative charge at the interface. To determine if subunit affinity is weaker at higher pH values, we measured the specific activity of F₂Y₃₅₆- $\beta 2$ at different protein concentrations (0.2–2 μ M, 5-fold excess $\alpha 2$) and at three different pH values (6.8, 7.6 and 8.2, data not shown). Similar specific activities were measured at higher protein concentrations indicating that any decrease in subunit affinity is minimal.

A second possible explanation for the loss of activity could be that PCET steps across the subunit interface are differentially affected by the charge change. Our recent high-field electron nuclear double resonance (ENDOR) spectroscopy experiments^{40, 41} suggest that RT across the interface is distinct from the orthogonal RT in β and co-linear RT in α . The impact of charge changes on the water clusters at the subunit interface remains to be determined. This alternative interpretation of the pH rate profiles thus remains a possibility.

Role of E₃₅₀ in initiating RT by reduction of Y₁₂₂•

Reaction of F₃Y₁₂₂•/E₃₅₀D- $\beta 2$, wt- $\alpha 2$, CDP and ATP does not accumulate Y₃₅₆•

—An alternative explanation for the inactivity observed with the E₃₅₀ mutants is that this residue plays an essential role in the protein conformational change that initiates RT.²⁶ This conformational change occurs upon substrate/effector binding to $\alpha 2$ and is proposed to be related to the initial PT step from the H₂O bound to FeI of the diferric cluster to Y₁₂₂•, which occurs concomitant with ET from Y₃₅₆ (Figure 1).¹⁵ We recently demonstrated that incubation of F₃Y₁₂₂•- $\beta 2$ with $\alpha 2$ /CDP/ATP generates Y₃₅₆• and dCDP with rate constants

of $20\text{--}30\text{ s}^{-1}$,¹² suggesting that this mutant allows partial uncoupling of the rate-limiting conformational gating ($\sim 5\text{ s}^{-1}$ in wt- $\beta 2$).²⁶ We speculated that partial removal of conformational gating with this probe might facilitate observation of $Y_{356}^{\bullet}$ in the reaction of $F_3Y_{122}^{\bullet}/E_{350}D\text{-}\beta 2$, $\alpha 2$, CDP and ATP.

The double mutant, $F_3Y_{122}^{\bullet}/E_{350}D\text{-}\beta 2$, was expressed, purified and reconstituted to assemble the diferric- $F_3Y_{122}^{\bullet}$ cofactor ($0.5\text{ }F_3Y^{\bullet}/\beta 2$). The protein was then incubated with wt- $\alpha 2$, CDP and ATP and the reaction was monitored from 16 to 535 ms by RFQ-EPR spectroscopy (Figure 5A). No changes in the total amount of radical or variations in the $F_3Y_{122}^{\bullet}$ spectral features were observed within this time frame. Subtraction of $F_3Y^{\bullet}$ from each composite spectrum (Figure 5B) revealed the spectrum in black (see expanded version in the inset), which does not resemble that of $Y_{356}^{\bullet}$,^{10–12} shown in Figure 5C. A direct comparison between the net spectrum (inset, Figure 5B) and that of $Y_{356}^{\bullet}$ is shown in Figure S6. These results contrast with studies with the single mutant $F_3Y_{122}^{\bullet}\text{-}\beta 2$, where 50% $Y_{356}^{\bullet}$ accumulates within 100 ms.¹² The data demonstrate that $F_3Y^{\bullet}$ is unable to oxidize Y_{356} when E_{350} is mutated.

Reaction of $NO_2Y_{122}^{\bullet}/E_{350}D\text{-}\beta 2$, wt- $\alpha 2$, CDP and ATP does not accumulate $Y_{356}^{\bullet}$ or $W^{+\bullet}$

The pathway radical $Y_{356}^{\bullet}$ was first observed in our lab in the reaction of $NO_2Y_{122}^{\bullet}\text{-}\beta 2$,¹¹ the most potent oxidant at position 122 investigated thus far. Detailed kinetic studies of $NO_2Y_{122}^{\bullet}\text{-}\beta 2/\alpha 2/CDP/ATP$ by stopped flow (SF) and RFQ-EPR methods revealed uncoupling of PT and ET steps during the initiation of the RT process. The phenolate of NO_2Y (NO_2Y^-), instead of the expected phenol, was formed with a rate constant of $100\text{--}300\text{ s}^{-1}$ and was accompanied by formation of a single dCDP with the same rate constant.¹¹ Unlike $F_3Y_{122}^{\bullet}\text{-}\beta 2$, multiple turnovers did not occur, as $Y_{356}^{\bullet}$ was unable to reoxidize NO_2Y^- upon reverse RT. The rapid rate constant for dCDP formation relative to wt- $\beta 2$ ($\sim 5\text{ s}^{-1}$)²⁶ suggests a more dramatic uncoupling of conformational gating and catalysis than was observed with $F_3Y_{122}^{\bullet}\text{-}\beta 2$. The mechanism of uncoupling in the two systems remains unknown.

We postulated that the reaction of $NO_2Y_{122}^{\bullet}/E_{350}D\text{-}\beta 2$, wt- $\alpha 2$, CDP and ATP might provide insight into conformational gating based on an experiment we designed to add to address the role of W_{48} , a residue long postulated to participate in the RT pathway (Figure 1).³ This study involved the reaction of $NO_2Y_{122}^{\bullet}/Y_{356}F\text{-}\beta 2$ in which the readily oxidized Y_{356} was replaced with the redox inert F. We hypothesized that substrate/effector (S/E or CDP/ATP) binding to the $NO_2Y_{122}^{\bullet}/Y_{356}F\text{-}\beta 2/\alpha 2$ complex might allow observation of $W_{48}^{\bullet}$ (or $W_{48}^{+\bullet}$) if the initiation of RT was successful. This reaction was investigated in detail by SF and RFQ-EPR spectroscopic methods (see SI for details). SF vis spectroscopy of the reaction showed S/E-dependent reduction of $NO_2Y^{\bullet}$ to NO_2Y^- concomitant with formation of a new species that has an absorption feature centered at 560 nm (Figure S7A–D, Table S5) and is produced in a kinetically competent fashion ($100\text{--}300\text{ s}^{-1}$). The 560 nm feature is similar to that of $W^{+\bullet}$ generated in water at pH 3.0 by pulse radiolysis (Figure S7C), supporting its identity as $W^{+\bullet}$.

The kinetics of this reaction were further studied by RFQ-EPR and RFQ-pulsed electron-electron double resonance (PELDOR) spectroscopic methods (Figures S8 and S9). The EPR

data reveal formation of multiple radical species on the same time scale as the 560 nm feature, though deconvolution of the spectra has remained a challenge and is ongoing. The PELDOR data further support formation of multiple radicals (Figure S9) and the analysis shows one distance measurement that could be associated $W_{48}^{+\bullet}$, the putative RT pathway residue. The data support formation of $W_{48}^{+\bullet}$, with the complexity associated with production of off-pathway radicals when the normal reductant of $NO_2Y_{122}^{\bullet}$, Y_{356} , is blocked.

The reactivity precedent observed with $NO_2Y_{122}^{\bullet}/Y_{356}F\text{-}\beta 2$ was the impetus to further interrogate the function of E_{350} . If E_{350} is only involved in accepting a proton from Y_{356} , then $NO_2Y_{122}^{\bullet}/E_{350}D\text{-}\beta 2$ should behave in a similar fashion to $NO_2Y_{122}^{\bullet}/Y_{356}F\text{-}\beta 2$, i.e. putative $W^{+\bullet}$ formation should be observed. If, however, E_{350} is required for the communication between the two subunits that initiates RT, then the $NO_2Y_{122}^{\bullet}/E_{350}D\text{-}\beta 2$ experiment would result in detection of only $NO_2Y_{122}^{\bullet}$.

The reaction of $NO_2Y_{122}^{\bullet}/E_{350}D\text{-}\beta 2$, $\alpha 2$, CDP and ATP was examined using a three-syringe mixing protocol (see methods section) due to the short half-life of $NO_2Y_{122}^{\bullet}$ (40 s at 25 °C). The EPR spectra of reactions quenched between 16 and 131 ms are shown in Figure 6A. No changes were observed in the total amount of radical ($\sim 1.2 NO_2Y^{\bullet}/\beta 2$) and no variations were seen in the spectral features of $NO_2Y_{122}^{\bullet}$. Subtraction of $NO_2Y^{\bullet}$ (blue, Figure 6B) from the composite spectrum at the 16 ms time point (pink, Figure 6B) reveals a net spectrum (black) that does not resemble $Y_{356}^{\bullet}$ (Figure 5C) or $W^{+\bullet}$ (Figure 6C). Similar results were observed for all time points. These results provide strong support for the requirement of E_{350} in the S/E triggered initiation of RT.

DISCUSSION

Rate-limiting protein conformational changes mask the long-range RT process (Figure 1) and nucleotide reduction chemistry in the *E. coli* class Ia RNR.²⁶ In the past decade, we have taken advantage of the technology to site-specifically incorporate UAAs to perturb the system such that the chemistry can be investigated.^{8, 10, 11, 14, 42} We have also recently developed photochemical RNRs in which a $[Re]^I$ photooxidant site-specifically attached at position 355 or 356 in $\beta 2$ can directly oxidize fluorinated derivatives of Y_{356} or Y_{731} , respectively which can oxidize subsequent Ys in the RT pathway in $\alpha 2$.^{43–46} The F_nY s ($n = 2$ or 3) are versatile probes to study PCET as their reduction potentials can be varied over 160 mV at pH 7.6 (25 mV easier to 135 mV harder to oxidize than Y)²⁴ and their pK_a s vary from 6.4 to 7.8.^{25, 35} The mutant proteins (F_nY -substituted RNRs and/or F_nY -photo-RNRs) have allowed the study of pathway radical intermediates by multifrequency EPR,⁴⁷ ENDOR⁴⁷ and transient absorption (TA) spectroscopic methods.⁴⁵ These tools have allowed demonstration of the reversibility of the 35 Å reduction/oxidation of $Y_{122}^{\bullet}$, and interrogation of the co-linear PCET process within $\alpha 2$,¹⁶ the initial orthogonal PCET step in $\beta 2$ proposed to play an important role in initiation of RT/conformational gating,¹⁵ and the putative role of water clusters at the α/β interface.⁴⁰ Additionally, photo-RNRs containing site-specifically incorporated F_nY s at position 356 have been used to measure ET rates within $\alpha 2$,⁴⁵ and the ET oxidation of F_nY_{356} within the α/β interface, which occurs in the Marcus inverted region.³⁹

With the exception of the first PCET step in RT proposed to involve ET from Y_{356} to $Y_{122}^{\bullet}$ coupled to PT from the water on $Fe1-H_2O$ (Figure 1), the PCET steps within $\beta 2$ have remained difficult to interrogate. In addition, despite the observation of $W_{48}^{+ \bullet}$ in the assembly of the essential diferric- $Y^{\bullet}$ cofactor,⁴⁸ no evidence currently exists for its involvement in the RT pathway. The importance of Y_{356} in the pathway has been established by studies in which 3,4-dihydroxyphenylalanine,⁸ 3-aminotyrosine²⁷ and $F_n Y^{13, 22}$ have been incorporated in place of Y_{356} , and by our more recent work with $NO_2 Y_{122}^{\bullet}-\beta 2^{11}$ and $F_3 Y_{122}^{\bullet}-\beta 2$.^{10, 12} However, the absence of structural insight about the location of this residue and consequently its positioning in the pathway relative to Y_{731} , has precluded a mechanistic understanding of the reversible oxidation process of these Ys across the α/β interface during turnover.

Sjöberg and coworkers identified the importance of E_{350} in catalysis by mutagenesis studies.²¹ The results led to their proposal that E_{350} was a key player in the communication between the two subunits. Subsequently, we suggested a specific role for E_{350} , deprotonation of Y_{356} during its oxidation.²² In the current paper, we have utilized four different UAA-substituted RNRs, $F_2 Y_{356}-\beta 2$, $F_2 Y_{731}-\alpha 2$, $F_3 Y_{122}^{\bullet}-\beta 2$ and $NO_2 Y_{122}^{\bullet}-\beta 2$ with different E_{350} mutations to provide evidence for its essential role in the initiation of RT.

Initially, the single mutants, $E_{350}X-\beta 2$ ($X = A, D$ or Q) were prepared and characterized. Despite having fully assembled cofactors, the A and Q mutants are inactive and the D mutant has very low activity. While these results demonstrate the importance of E_{350} , they preclude its functional interrogation. Our previous results with EPL-generated $2,3-F_2 Y_{356}-\beta 2^{13}$ suggested that it might be possible to address the role of E_{350} by monitoring the steady state activities of $F_2 Y_{356}-\beta 2$ and $F_2 Y_{731}-\alpha 2$ as a function of pH in the absence or presence of E_{350} . The data presented in Figure 4 demonstrate that both $F_2 Y$ -substituted proteins possess substantial activity over the entire pH regime and suggest that RT through either position can occur via conformationally gated ET. This hypothesis predicts that $E_{350}X/F_2 Y_{356}-\beta 2$ (or $E_{350}X-\beta 2/F_2 Y_{731}-\alpha 2$) would still maintain activity at alkaline pH via ET, if indeed the sole function of E_{350} is as a proton acceptor for one of the two pathway Ys (Y_{356} or Y_{731}). In addition, when $F_2 Y$ is $> 90\%$ protonated, the double mutants should be inactive, as no proton acceptor is present to deprotonate it. As seen in Figure 4 and Table S2, $E_{350}X/F_2 Y_{356}-\beta 2$ and $E_{350}D-\beta 2/F_2 Y_{731}-\alpha 2$ are inactive over the entire pH regime. Similar experiments performed with $F_2 Y_{356}-\beta 2/F_2 Y_{731}-\alpha 2$ revealed no measureable activity for the $E_{350}Q$ mutant at low or high pH. Unfortunately, the essential role of E_{350} in conformational gating has masked its potential role as a proton acceptor during oxidation of Y_{356} or Y_{731} . A definitive conclusion about E_{350} 's function with respect to PCET is thus not possible. Two types of experiments are ongoing which will address this potential role. In one experiment, we are using photo- $\beta 2$ s with a photooxidant attached to residue 355, $E_{350}Q$ and $F_n Y$ s at 356 to measure the pH dependence of radical injection and dCDP formation in $\alpha 2$ on a nanosecond time scale. In a second experiment, high-field 1H and 2H ENDOR and EPR spectroscopy at 263 GHz allow us to look at the H bonding network to $Y_{356}^{\bullet}$.^{40, 41}

Interpretation of pH rate profiles are notoriously complex and alternative proposals from the data presented above, including one in which the pK_2 s (Table S4) are indicative of the pK_a of $F_n Y$ is also possible. However, the pH rate profiles of $F_n Y$ s (3,5; 2,3,5; and 2,3)

incorporated into each pathway position (356- β , 731 and 730- α , data not shown) are the basis for our current interpretation.

Two additional experiments were designed to test if the importance of E₃₅₀ stems from its involvement in the conformational changes that trigger RT. In these studies we took advantage of the ability to perturb the reduction potential of Y₁₂₂ using NO₂Y₁₂₂ or F₃Y₁₂₂, which partially uncouple the conformational gating of RT based on the rate constants measured for Y₃₅₆[•] and dCDP formation in the reaction with α 2/CDP/ATP. These rate constants are 10 (F₃Y₁₂₂)¹² to 30-fold (NO₂Y₁₂₂)¹¹ greater than the rate constants measured for the conformational change in wt- β 2 (~5 s⁻¹).²⁶ The inability to observe Y₃₅₆[•] in F₃Y₁₂₂[•]/E₃₅₀D- β 2 under turnover conditions indicates that E₃₅₀ is important for RT, but does not eliminate its involvement in PT. However, we predicted that NO₂Y₁₂₂[•]/E₃₅₀D- β 2 might behave in a similar fashion to NO₂Y₁₂₂[•]/Y₃₅₆F- β 2 in a reaction with α 2/CDP and ATP; in the latter case we have shown rapid formation of multiple radicals (Figures S7–9). While the identity of the radicals remain unassigned, both the visible spectrum (Figure S7C) and one of the distances observed by the PELDOR analysis (Figure S9B) suggest that W₄₈⁺ in β 2 accounts for 29% of the total radical observed. Currently we interpret these data as reporting on the formation of off-pathway radicals by the strong oxidant NO₂Y₁₂₂[•], although generation of these species still requires the presence of CDP/ATP. Our inability to detect formation of any analogous radicals with NO₂Y₁₂₂[•]/E₃₅₀D- β 2, α 2, CDP and ATP suggests that the active enzyme conformation that initiates the RT process does not occur in this mutant, supporting a structural role for E₃₅₀ in the initiation of RNR catalysis.

The molecular basis by which E₃₅₀ controls the conformationally triggered RT remains to be unraveled. However, the observations reported herein suggest that conserved, charged residues located at the α/β subunit interface may play a very important role in this process. Several residues have been identified by sequence alignments⁴⁹ and are currently being investigated in the context of the α 2 β 2 docking model. The importance of charged residues and electrostatics in ET across protein interfaces has recently been highlighted by the beautiful studies on ET between the physiological redox protein partners, myoglobin and cytochrome b₅.⁵⁰ The affinity between these two proteins is weak (50–200 μ M).⁵¹ By redesigning their interface with the addition of three lysines, the authors reported the fastest ET rates yet observed between the two proteins. Thus, minimizing the surface interaction space and distance for this ET process resulted in enhanced rates. The interactions between the RNR subunits are also weak (0.2 μ M rather than 50–200 μ M). However, this process in RNR likely requires a much more carefully orchestrated surface interaction to permit the proton coupling in the ET process between Y₃₅₆ in β 2 and Y₇₃₁ in α 2.

In a subsequent paper, we will show that E₅₂X- β 2 (X = A, D or Q) and R₃₂₉X- α 2 (X = A or Q) affect α/β subunit interactions, similar to the behavior reported herein for E₃₅₀. We believe that the data presented in this paper strongly support the role of E₃₅₀ in conformational gating, a feature long known to be important in RNR catalysis, but about which we currently lack molecular understanding.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

$\alpha 2$	RNR large subunit
$\beta 2$	RNR small subunit
E	effector
ET	electron transfer
F₂Y	3,5-difluorotyrosine
F₃Y	2,3,5-trifluorotyrosine
NO₂Y	3-nitrotyrosine
PCET	proton-coupled electron transfer
PT	proton transfer
RNR	ribonucleotide reductase
RT	radical transport
S•	cysteine radical
S	substrate
TR	thioredoxin
TRR	thioredoxin reductase
UAA	unnatural amino acid
wt	wild-type
Y•	tyrosyl radical

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FIGURE 1.

The proposed RT pathway in *E. coli* class Ia RNR.^{3, 7} The pathway consists of the conserved residues: Y₁₂₂ and Y₃₅₆ in β 2, and Y₇₃₁, Y₇₃₀, C₄₃₉ in α 2. The direction of movement of the electron and the proton are represented by the pink and purple arrows, respectively. The dotted lines represent steps for which there is no direct experimental evidence. W₄₈ and its putative proton acceptor D₂₃₇ are shown in gray, as there is no direct experimental evidence for their involvement in the RT pathway. Y₃₅₆ and E₃₅₀ are highlighted in blue as their locations cannot be determined from X-ray structures of β 2.

FIGURE 2.

Probes to investigate the role of E₃₅₀ in RNR function. A reductionist view of the RT pathway showing only the key amino acids in each experiment. A–C. F₂Y as a probe for PT using E₃₅₀X (X = A, D or Q). These experiments used F₂Y₃₅₆, F₂Y₇₃₁ or F₂Y at both 356 and 731. The activities of these mutant enzymes were tested as a function of pH. D–E. F₃Y₁₂₂•-β₂ and NO₂Y₁₂₂•-β₂ as probes for the role of E₃₅₀ in conformational change(s). E₃₅₀ was mutated to D in these experiments, and the reaction was monitored by RFQ-EPR spectroscopy to look for formation of Y₃₅₆• or W⁺.

FIGURE 3.

The pH rate profiles of wt- and F₂Y-substituted RNRs. A. The specific activities of wt-β2 (purple) and F₂Y₃₅₆-β2 (pink) as a function of pH. β2 was assayed in the presence of a 5-fold excess of wt-α2. B. The specific activities of wt-α2 (blue) and F₂Y₇₃₁-α2 (green) as a function of pH. α2 was assayed in the presence of a 5-fold excess of wt-β2. C. The specific activities from panels A and B replotted as the percent maximum measured activity for each subunit. Re-analysis of the data in this manner reveals that although the absolute specific activities differ between subunits, the pH rate profiles of wt-α2 and wt-β2 are identical, as are the pH rate profiles of F₂Y₃₅₆-β2 and F₂Y₇₃₁-α2. The black lines in each panel represent fits to a two-proton ionization model (Eq. 2). Data points represent either a single trial (green) or the averages of two independent trials (purple, pink, and blue).

FIGURE 4.

The pH rate profiles of E₃₅₀D(A)/F₂Y₃₅₆-β₂ and E₃₅₀D-β₂/F₂Y₇₃₁-α₂ determined by the radioactive assay for RNR. A. Measured specific activities of wt-β₂ (purple), 3,5-F₂Y₃₅₆-β₂ (pink), E₃₅₀D/F₂Y₃₅₆-β₂ (blue) and E₃₅₀A/F₂Y₃₅₆-β₂ (green). The percentage of F₂Y₃₅₆⁻ as a function of pH is shown by the orange dots. Data points represent either a single trial (blue, green) or the averages of two independent trials (purple, pink). Expanded views of the E₃₅₀D(A)/F₂Y₃₅₆-β₂ pH rate profiles are shown in Figure S5A and S5B. B. The pH rate profile of wt-β₂/wt-α₂ (purple), wt-β₂/F₂Y₇₃₁-α₂ (pink) and E₃₅₀D-β₂/F₂Y₇₃₁-α₂ (blue). The percentage of F₂Y₇₃₁⁻ as a function of pH is shown in orange. Data points represent either a single trial (pink) or the averages of two independent trials (purple, blue). An expanded view of the E₃₅₀D-β₂/F₂Y₇₃₁-α₂ pH rate profile is shown in Figure S5C.

FIGURE 5.

Reaction of $F_3Y_{122}^{\bullet}/E_{350}D\text{-}\beta 2$, wt- $\alpha 2$, CDP and ATP monitored by RFQ-EPR spectroscopy.

A. The composite EPR spectra recorded at the indicated time points. B. Subtraction of $F_3Y^{\bullet}$ (blue) from the composite spectrum at 16 ms (pink) reveals the spectrum in black (inset).

C. Spectrum of $Y_{356}^{\bullet}$ observed in the reaction of $F_3Y_{122}^{\bullet}\text{-}\beta 2$, $\alpha 2$, CDP and ATP.^{10, 12} A spectral overlay between the black inset in panel B and $Y_{356}^{\bullet}$ is shown in Figure S6.

FIGURE 6.

Reaction of $\text{NO}_2\text{Y}_{122}\bullet/\text{E}_{350}\text{D}-\beta 2$, wt- $\alpha 2$, CDP and ATP monitored by RFQ-EPR spectroscopy. A. The composite EPR spectra collected at the indicated time points. B. Subtraction of $\text{NO}_2\text{Y}\bullet$ (blue) from the composite spectrum at 16 ms (pink) produces the spectrum in black (inset). C. Spectrum of the putative $W^{+\bullet}$ radicals recorded in the reaction of $\text{NO}_2\text{Y}_{122}\bullet/\text{Y}_{356}\text{F}-\beta 2$, wt- $\alpha 2$, CDP and ATP (Figure S8B). The spectrum of $\text{Y}_{356}\bullet$ generated by the single mutant ($\text{NO}_2\text{Y}_{122}\bullet-\beta 2$) is identical to that shown in Figure 5C.¹¹ A spectral overlay between the black inset in panel B and the new radicals ($W^{+\bullet}$) is shown in Figure S10.

TABLE 1Specific activities of E₃₅₀X-β2 and K_d for α2/E₃₅₀X-β2 subunit interaction.

Sample	K _d (μM) ^a	Specific activity ^b (nmol/min/mg)	Y•/β2	% wild type activity
wt-β2	0.18 ^c	6750 ± 100	1.2	-
E ₃₅₀ A-β2	2.2 ± 0.2	11 ± 1	1.0	0.17
E ₃₅₀ D-β2	1.7 ± 0.1	29 ± 0.1	1.1	0.43
E ₃₅₀ Q-β2	2.2 ± 0.3	16 ± 1	1.1	0.23

^aDetermined using the competitive inhibition assay.²³^bDetermined using the radioactive assay.³²^cFirst reported by Sjöberg and coworkers.²³

TABLE 2Specific activity of E₃₅₀Q/F₂Y₃₅₆-β₂ with F₂Y₇₃₁-α₂.

β ₂	α ₂	pH	Specific activity (nmol/min/mg)
wt	F ₂ Y ₇₃₁	6.8	490 ^a
wt	F ₂ Y ₇₃₁	8.4	720 ^a
F ₂ Y ₃₅₆	wt	6.8	3300 ^a
F ₂ Y ₃₅₆	wt	8.4	2400 ± 140 ^a
F ₂ Y ₃₅₆	F ₂ Y ₇₃₁	6.8	810 ± 30 ^b
F ₂ Y ₃₅₆	F ₂ Y ₇₃₁	8.4	1660 ± 30 ^b
E ₃₅₀ Q/F ₂ Y ₃₅₆	F ₂ Y ₇₃₁	6.8	_ ^c
E ₃₅₀ Q/F ₂ Y ₃₅₆	F ₂ Y ₇₃₁	8.4	_ ^c

^aDetermined by the radioactive assay.³²^bDetermined by the spectrophotometric assay.⁵²^cThe measured slope was indistinguishable from the background slope observed in a negative control assay lacking β₂.