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Citation

As Published
http://dx.doi.org/10.1021/bi101319v

Publisher
American Chemical Society (ACS)

Version
Author's final manuscript

Accessed
Mon Dec 31 02:18:09 EST 2018

Citable Link
http://hdl.handle.net/1721.1/72158

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Use of 2,3,5-F$_3$Y-$\beta$2 and 3-NH$_2$Y-$\alpha$2 to study PCET in E. coli Ribonucleotide Reductase

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Abstract

E. coli ribonucleotide reductase is an $\alpha_2\beta_2$ complex that catalyzes the conversion of nucleoside 5'-diphosphates (NDPs) to deoxynucleotides (dNDPs). The active site for NDP reduction resides in $\alpha_2$, and the essential diferric-tyrosyl radical (Y$_{122}$•) cofactor that initiates radical transfer to the active site cysteine in $\alpha_2$ (C$_{439}$), 35 Å removed, is in $\beta_2$. The oxidation is proposed to involve a hopping mechanism through aromatic amino acids (Y$_{122}$•→W$_{48}$→Y$_{356}$ in $\beta_2$ to Y$_{731}$→Y$_{730}$→C$_{439}$ in $\alpha_2$) and reversible proton coupled electron transfer (PCET). Recently 2,3,5-F$_3$Y (F$_3$Y) was site-specifically incorporated in place of Y$_{356}$ in $\beta_2$, and 3-NH$_2$Y (NH$_2$Y) in place of Y$_{731}$ and Y$_{730}$ in $\alpha_2$. A pH rate profile with F$_3$Y$_{356}$-$\beta_2$ suggested that as the pH is elevated, the rate-determining step of RNR can be altered from a conformational change to PCET and that the altered driving force for F$_3$Y oxidation, by residues adjacent to it in the pathway, is responsible for this change. Studies with NH$_2$Y$_{731(730)}$-$\alpha_2$/CDP/ATP resulted in detection of NH$_2$Y radical (NH$_2$Y•) intermediates capable of dNDP formation. In this study, the reaction of F$_3$Y$_{356}$-$\beta_2$/CDP/ATP has been examined by stopped flow (SF) absorption and rapid freeze quench EPR spectroscopy and has failed to reveal any radical intermediates. F$_3$Y$_{356}$-$\beta_2$/CDP/ATP has also been examined with NH$_2$Y$_{731}$-$\alpha_2$ (or NH$_2$Y$_{730}$-$\alpha_2$) by stopped-flow kinetics from pH 6.5–9.2 and revealed rate constants for NH$_2$Y• formation that support a change in rate limiting step at elevated pH. The results together with kinetic simulations provide a guide for future studies to detect radical intermediates in the pathway.

Ribonucleotide reductases (RNRs) are responsible for reduction of nucleotides to 2'-deoxyribonucleotides (dNDPs), supplying the precursors required for DNA replication and repair (1,2). Active E. coli RNR is a 1:1 complex of two homodimeric subunits: $\alpha_2$ and $\beta_2$ (3-5). $\alpha_2$ harbors the active site, where thyl radical-mediated (C$_{439}$•) nucleotide reduction occurs (6-8), and the binding sites for allosteric effectors, which control the rate and specificity of reduction (9). $\beta_2$ houses the essential diferric-tyrosyl radical (Y$_{122}$•) cofactor (10,11). Each turnover requires oxidation of C$_{439}$ in $\alpha_2$ by Y$_{122}$• in $\beta_2$ (12). A structure of the active $\alpha_2\beta_2$ complex is unavailable. However, a docking model of this complex, based...
on shape complementarity of the structures of the individual subunits, led Uhlin and Eklund to propose that radical transfer between subunits occurs over a distance of 35 Å by a pathway involving aromatic amino acid radicals (Fig. 1A, B) (13). Mutagenesis studies suggested that the residues shown in Figs. 1A and 1B are important for catalysis (14-16).

The inability of the mutants, however, precluded mechanistic studies. We have recently suggested that the residues shown in Figs. 1A and 1B are important for catalysis (14-16). The proposed pathway (Fig. 1A) has been further explored by replacing Y356, Y731 and Y730 (17-19), the pathway dependence (20), and the docking model (21).

In the present study, we provided direct evidence for the three proposed redox-active tyrosines (Yincorporated unnatural amino acids site-specifically into the pathway. Our studies have suggested that the residues shown in Figs. 1A and 1B are important for catalysis (14-16). The inactivity of the mutants, however, precluded mechanistic studies. We have recently suggested that the residues shown in Figs. 1A and 1B are important for catalysis (14-16). The proposed pathway (Fig. 1A) has been further explored by replacing Y731 and Y730 in α2 with NH2Y, a tyrosine analog that is easier to oxidize than Y by 190 mV at pH 7 (Fig. 1C).

α in these studies has a single mutation: Y to NH2Y (27). SF experiments in the presence of β2, CDP and ATP revealed that an NH2Y radical (NH2Y•) is generated in a kinetically competent fashion in two phases with rate constants of 18 s⁻¹ and 2.5 s⁻¹ with Y730NH2Y-α2 and of 12 s⁻¹ and 2.5 s⁻¹ with Y730NH2Y-α2 at pH 7.6 (19). These studies and more recent...
studies (Minnihan, Seyedsayamdost & Stubbe, manuscript in preparation) have further revealed that these mutants can make dNDPs at rates of 0.3–0.6 s\(^{-1}\), substantially slower than the rates of NH\(_2\)Y• formation.

In the present paper, F\(_3\)Y\(_{356}\)-β\(_2\) was examined with α2/CDP/ATP to look for radical intermediates by SF and RFQ EPR spectroscopy and none were detected. The hypothesis that an E\(_p\) difference of >80 mV between F\(_3\)Y and Y changes the rate-determining step of RNR from conformational gating to a step or steps involved in PCET has also been examined: F\(_3\)Y-β\(_2\) was incubated with NH\(_2\)Y\(_{731}\)-α2 or NH\(_2\)Y\(_{730}\)-α2 and the pH rate profile of NH\(_2\)Y• formation from pH 6.5 to 9.2 was investigated by SF absorption spectroscopy. The rate constants for NH\(_2\)Y• formation are triphasic. Despite the kinetic complexity, they reveal that the pH rate profile for NH\(_2\)Y• formation is very similar to that observed previously in the steady state for dNDP formation with F\(_3\)Y\(_{356}\)-β\(_2\)/α2/CDP/ATP and are distinct from the steady state data with intein wt-β\(_2\) control. A kinetic model to accommodate these results is presented that supports the proposed shift in the rate-limiting step and the kinetic simulations provide an explanation for why transient radical intermediates are not detected. This model provides a framework for future studies involving F\(_n\)Y incorporation using evolved tRNA/tRNA synthetase pairs, and addressing the importance of W\(_{48}\) in the pathway (Figs. 1A & 1B).

Materials and Methods

Materials

[2-\(^{14}\)C]-CDP (50 μCi/mL) was purchased from Moravek Biochemicals and calf-intestine alkaline phosphatase (20 U/μL) was from Roche. Lithium 8-hydroxyquinoline-6-sulfonate and Sephadex G-25 were from Sigma. 2-[N-morpholino]-ethanesulfonic acid (Mes), N-2-hydroxethylpiperazine-N’-2-ethanesulfonic acid (Hepes), N-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid (Taps), 2-[cyclohexylamino]-ethanesulfonic acid (Ches) and Emulsifier-Safe scintillation liquid were obtained from EMD Bioscience. Slide-a-lyzer cassettes were from Pierce. α2 was expressed, purified, and pre-reduced as reported and had a specific activity of 2500 nmol/min mg (18,28). E. coli thioredoxin (TR, 40 U/mg) (29) and TR reductase (TRR, 1800 U/mg) were isolated as previously described (30). Y\(_{730}\)NH\(_2\)Y-α2 and Y\(_{731}\)NH\(_2\)Y-α2 were isolated as previously described and had specific activities of 100 and 175 nmol/min mg, respectively (19).

Semisynthesis of F\(_3\)Y\(_{356}\)-β\(_2\)

Generation of F\(_3\)Y\(_{356}\)-β\(_2\) by EPL and its purification were carried out as detailed previously (31).

Generation of apo F\(_3\)Y\(_{356}\)-β\(_2\) with lithium 8-hydroxyquinoline-6-sulfonate

The apo form of intein wt-β\(_2\) and F\(_3\)Y\(_{356}\)-β\(_2\) were generated by a modification of the procedure of Atkin et al (32). Briefly, a solution of 2.5 mL of each β\(_2\) variant (~20 mg, 90 μM) was dialyzed against 500 mL of chelator solution consisting of 1 M imidazole, 30 mM NH\(_3\)OH, 50 mM 8-hydroxyquinoline-6-sulfonate (pH 7.0) in a 3 mL Slide-a-lyzer cassette for 3 h. The chelator was then removed by dialysis against 4 L Hepes buffer (50 mM Hepes, 5 % glycerol, pH 7.6) for 3 h and further by desalting on a Sephadex G 25 column (1.5 × 23, 40 mL) equilibrated in Hepes buffer. This procedure gives the apo form of each β\(_2\) variant in 80–95 % yield. The concentration of apo β\(_2\) was determined using ε\(_{280\ nm}\) = 120 mM\(^{-1}\)cm\(^{-1}\).
Reconstitution of apo β2

The apo form of each β2 variant was reconstituted as reported previously. This procedure yields a radical content of ∼1.2 Y_{122}\beta2 as determined by the dropline procedure (33).

Spectrophotometric and radioactive RNR Assays

RNR activity assays were performed as previously described (18). The final concentration of α2 and β2 variants in these assays were each 3 μM. The specific activity of [2\,^{14}\text{C}] -CDP was 3800 cpm/nmol.

Single Wavelength and Diode Array SF Absorption Spectroscopy

SF absorption kinetics were performed on an Applied Photophysics DX. 17MV instrument equipped with the Pro-Data upgrade. The temperature was maintained at 25°C with a Lauda RE106 circulating water bath. Single wavelength kinetics experiments utilized PMT detection at 410 nm (λ_{max} of Y_{122}• with ε = 3700 M^{-1}cm^{-1}) (33), 510 nm (λ_{max} of W• with ε = 2200 M^{-1}cm^{-1}) and 560 nm (λ_{max} of WH•+ with ε = 3000 M^{-1}cm^{-1}) (34). Typically, pre-reduced α2 (50–70 μM) and ATP (6 mM) in one syringe were mixed with F_3Y_{356}\beta2 (50–70 μM) and CDP (2 mM) in a 1:1 ratio in 50 mM Taps, 15 mM MgSO_4, 1 mM EDTA, pH 8.4. Time courses shown are the average of at least 5 individual traces. Diode array SF absorption spectroscopy was carried out with an Applied Photophysics PDA.1 Photodiode Array detector. The concentration of the reaction components were the same as described for single wavelength kinetics.

RFQ EPR Spectroscopy

RFQ EPR samples were prepared using an Update Instruments 1019 Syringe Ram Unit, a Model 715 Syringe Ram Controller and a quenching bath. The temperature of the liquid isopentane bath was controlled with a Fluke 52 Dual Input Thermometer, equipped with an Anritsu Cu Thermocouple probe for the isopentane bath and the funnel. Stainless steel packers were purchased from McMaster-Carr and were cut to a length of 40 cm and deburred at the MIT machine shop. The dead-time of the set-up was determined to be 16 ± 2 ms with two independent measurements of the myoglobin/NaN_3 test reaction. A packing factor of 0.60 ± 0.05 was reproducibly obtained as tested with intein-wt β2 samples. Routinely, a ram push velocity of 1.25 or 1.6 cm/s was used and the displacement was adjusted to expel 300 μL sample after the reaction.

Operation of the apparatus was similar to the procedure previously described (35). Typically, pre-reduced α2 (50–70 μM) and ATP (6 mM) in one syringe were mixed with F_3Y_{356}\beta2 and CDP (2 mM) in the second syringe in a 1:1 ratio in 50 mM Taps, 15 mM MgSO_4, 1 mM EDTA, pH 8.4. When the temperature of the EPR tube-funnel assembly had equilibrated to the bath temperature, the contents of each syringe were mixed rapidly in a mixing chamber and aged for a pre-determined time period by pushing the contents through a reaction loop. The sample was sprayed into the EPR tube-funnel assembly which was held at a distance of ≤1 cm from the spray nozzle. The assembly was immediately returned to the bath and the crystals allowed to settle for 15–30 s. The sample was then packed into the EPR tube using the stainless steel packers described above.

EPR spectra were recorded at the Department of Chemistry Instrumentation Facility on a Bruker ESP-300 X-band (9.4 GHz) spectrometer. Spectra at 77 K were recorded with a quartz finger dewar filled with liquid N_2; spectra at 15 K were acquired with an Oxford liquid helium cryostat and an Oxford ITC 503 temperature controller. Unless noted otherwise, EPR parameters were as follows: Power = 50 μW, modulation amplitude = 1.5 G, modulation frequency = 100 kHz, time constant = 5.12 ms and scan time = 41.9 s.
pH Rate Profile of NH$_2$Y$^*$ Formation in the Reaction of F$_3$Y$_{356}$-β2 with NH$_2$Y-α2s monitored by SF-Absorption Spectroscopy

NH$_2$Y-α2 was prepared and pre-reduced as detailed previously (19). In all experiments, pre-reduced NH$_2$Y-α2 and ATP were mixed with F$_3$Y$_{356}$-β2 and CDP to yield final concentrations of 4 μM, 3 mM, 4 μM and 1 mM, respectively. Single wavelength kinetics were monitored using PMT detection at 320 nm ($\lambda_{\text{max}}$ of NH$_2$Y$_{731}^•$ with ε = 11000 M$^{-1}$cm$^{-1}$) or 325 nm ($\lambda_{\text{max}}$ of NH$_2$Y$_{730}^•$ with ε = 10500 M$^{-1}$cm$^{-1}$) (19). Reactions were carried out in 15 mM MgSO$_4$, 1 mM EDTA and 50 mM Goods Buffers [Mes (pH 6.5–7), Hepes (pH 7–8), Taps (pH 8–8.8) or Ches (pH 8.8–9.2) buffer] adjusted to the desired pH. Syringes and reaction lines were equilibrated in the desired buffer prior to the experiment. At each pH, 6–8 traces were averaged and analyzed using OriginPro Software. Iterative rounds of fitting were carried out until the R$^2$ value was maximized (≥0.99) and the residual plot was randomly scattered around zero ± 0.001 AU.

Results

Pre-steady State Experimental Design

Previous pre-steady state examination of wt RNR at pH 7.6 has shown that production of dCDP is rate-limited by a conformational change that precedes radical transfer, resulting in a burst of dCDP formation in the first turnover with rate constants of 4.4–10 s$^{-1}$ (36). In wt RNR, this conformational change(s) kinetically masks detection of the proposed aromatic amino acid radical intermediates during radical transfer (Figs. 1A & 1B). Thus, detection of transient radical intermediates requires, at a minimum, that the rate constant for radical transfer be diminished relative to that for the conformational step. Our previous results with F$_3$Y-β2s suggested that insertion of F$_3$Y in place of Y$_{356}$ provides a sufficient shift in the driving force for radical propagation, to make it rate-limiting at elevated pH (18). Thus it is possible that pathway radical intermediates could be detected by RFQ EPR methods at elevated pH.

Increasing the Y$_{122}^*$ Radical Content of Semisynthetic F$_3$Y$_{356}$-β2

To maximize our chances of detecting low levels of pathway radical intermediates, we focused on increasing the concentration of Y$_{122}^•$ in the semisynthetic β2s using the method of Atkins et al. (32). With both intein wt-β2 and F$_3$Y$_{356}$-β2, we were able to obtain ~1.2 Y$_{122}^•$/β2 with good protein recoveries (80 to 95 %). The U/V-vis spectrum, EPR spectrum, and SDS PAGE of these proteins are shown in Figs. S1 and S2. The spectra are identical to those of recombinant wt-β2 indicating an intact diferric-Y$_{122}^•$ cofactor. Assays for dCDP production before and after application of this procedure, gave specific activities that correlated with Y$_{122}^•$ content. Thus, the procedure increased the radical content of F$_3$Y-β2 and elevated nucleotide reduction activity proportionally.

SF Absorption and RFQ EPR Spectroscopies with F$_3$Y$_{356}$-β2/α2

To test whether radical intermediates could be detected during the radical propagation process with F$_3$Y$_{356}$-β2 at pH 8.4, where this process is proposed to be rate-limiting, SF absorption and RFQ EPR studies were carried out with wt α2. Using the $E_{0s}$ measured for the blocked amino acids, W, F$_3$Y, Y and NH$_2$Y (Fig. 1C), an energy landscape for the pathway (Fig. 1D) was created to help visualize how insertion of F$_3$Y into the pathway and pH might alter the ability of adjacent residues in the pathway to mediate its oxidation. If forward radical transfer is slow at pH 8.4 for example, then a neutral W$_{38}$ could build up, if it is not rapidly reduced by Y$_{122}^•$. Ws have reported $\lambda_{\text{max}}$s from 485–530 nm (ε = 1750–2300 M$^{-1}$cm$^{-1}$). We also considered the possibility that WH•+ might be observed and they have reported $\lambda_{\text{max}}$ between 560–600 (ε = 2500–2900 M$^{-1}$cm$^{-1}$) (34,37). If reverse radical
transfer is slow, then the species most likely to build up at pH 8.4 is the Y₇₃₁•, based on $E_P$

difference of 110 mV relative to F₃Y (Figs. 1C & 1D). Y•s have $\lambda_{max}$s in the range of 407–
410 ($\epsilon = 2750$–$3200 \text{ M}^{-1} \text{cm}^{-1}$) (38,39). The results from SF absorption experiments

are presented in Fig. 2A. They show that no changes are observed at 410 nm ($\lambda_{max}$ for Y₁₂₂•),
510 nm or 560 nm. The minor changes that are observed are likely related to small structural
perturbations associated with the di-iron cluster upon binding to o₂ (20). SF diode array
absorption spectroscopy also failed to reveal any changes in the region of 400-800 nm (data
not shown). Under these experimental conditions, no absorption features associated with a
W• or WH•+ were apparent.

As mentioned above, the absorption features associated with the Y• range from 407–410 nm
with varying degrees of sharpness. The Y₁₂₂• and Y₇₃₁• features are thus likely to be
similar, making build up of a transient Y₇₃₁• difficult to distinguish from Y₁₂₂• by vis
spectroscopy. The EPR features of Y₁₂₂• and Y₇₃₁•, on the other hand, are highly dependent
on the dihedral angle of the $\beta$ protons relative to the aromatic ring (40,41). Based on the
crystal structures of o₂ and β₂, Y₁₂₂, Y₇₃₁ and Y₇₃₀ have dihedral angles of ~90°, 33° and
37°, respectively (13), making it feasible that the EPR spectra of the latter two would be
distinct from that of Y₁₂₂• (42). In accord with the structural data, we recently determined a
dihedral angle of 46° for NH₂Y₇₃₀• by EPR analysis (43). Consequently, RFQ-EPR studies
were carried out under conditions similar to those in the SF absorption experiments to look
for a new Y•. The reaction was quenched from 28 to 1912 ms. The traces obtained at 72, 138
and 1912 ms are shown in Fig. 2B and those at 28 and 612 ms in Fig. S3. EPR analysis and
spin quantitation at 77 K or 15 K at all time points showed that the [Y•] observed is identical
with varying degrees of sharpness. The Y• formation resulting due to the ease of its oxidation. The slower rate
constants were proposed to be associated with the rate limiting conformational change

responsible for dNDP production under steady state conditions. NH₂Y• formation was
“complete” within 20 s and its concentration remained unchanged for several minutes. The
decay of NH₂Y• is slow with a rate constant of 0.0062 ± 0.0012 for NH₂Y₇₃₁• (Fig. S4) and
0.0043 ± 0.0011 s⁻¹ for NH₂Y₇₃₀• (Fig. S5). Finally both 730 and 731 NH₂Y mutants
supported dCDP formation with rate constants of 0.3–0.6 s⁻¹, substantially lower than the
rate constant of NH₂Y• production (Minnihan, Seyedsayamdost & Stubbe, manuscript in
preparation). These rate constants would be further reduced with F₃Y₅₅₆•β₂, as intein wt-β₂
has 25 % the activity of wt-β₂. These observations together suggest that under the conditions
of the SF experiments described subsequently, NH₂Y can function as a radical trap reporting
on the rate constant for forward radical propagation.

**Use of NH₂Y-α2 as a Reporter of Forward Radical Transfer with F₃Y₅₅₆•β₂**

Our previous SF studies at pH 7.6 with β₂, Y₇₃₁NH₂Y-α2 (or Y₇₃₀NH₂Y-α2), CDP/ATP
revealed that NH₂Y• is formed with biphasic kinetics and rate constants of 18 s⁻¹ and 2.5
s⁻¹ (12 s⁻¹ and 2.5 s⁻¹) (19). The faster rate constants were proposed to be associated with
electron delocalization within the protein in a non-productive conformation for nucleotide
reduction, with NH₂Y• formation resulting due to the ease of its oxidation. The slower rate
constants were proposed to be associated with the rate limiting conformational change

responsible for dNDP production under steady state conditions. NH₂Y• formation was
“complete” within 20 s and its concentration remained unchanged for several minutes. The
decay of NH₂Y• is slow with a rate constant of 0.0062 ± 0.0012 for NH₂Y₇₃₁• (Fig. S4) and
0.0043 ± 0.0011 s⁻¹ for NH₂Y₇₃₀• (Fig. S5). Finally both 730 and 731 NH₂Y mutants
supported dCDP formation with rate constants of 0.3–0.6 s⁻¹, substantially lower than the
rate constant of NH₂Y• production (Minnihan, Seyedsayamdost & Stubbe, manuscript in
preparation). These rate constants would be further reduced with F₃Y₅₅₆•β₂, as intein wt-β₂
has 25 % the activity of wt-β₂. These observations together suggest that under the conditions
of the SF experiments described subsequently, NH₂Y can function as a radical trap reporting
on the rate constant for forward radical propagation.

**SF Absorption Spectroscopy with F₃Y₅₅₆•β₂/NH₂Y-α2/CDP/ATP**

SF experiments were thus carried out with F₃Y₅₅₆•β₂ and NH₂Y₇₃₀•α2 or NH₂Y₇₃₁•α2. The
final concentration of protein in these experiments was 4 μM, similar to those previously

Biochemistry. Author manuscript; available in PMC 2012 March 1.
reported in the steady state pH rate profile studies (3 μM) (36). The ability to generate 1.2 Y\textsubscript{122\*} per Y\textsubscript{356}F\textsubscript{3}Y-β2, greatly facilitated the analysis.

The results of the pH-dependent SF experiments with NH\textsubscript{2}Y\textsubscript{730-α2} (or NH\textsubscript{2}Y\textsubscript{731-α2}) and F\textsubscript{3}Y\textsubscript{356}β2 are shown in Fig. 3. Expanded views of the first several seconds of each trace are shown in Fig. S6 (for Y\textsubscript{731}NH\textsubscript{2}Y-α2) and Fig. S7 (for Y\textsubscript{730}NH\textsubscript{2}Y-α2). Reactions monitored for 20 s at pH 6.5, 8.45 and 8.6 (for Y\textsubscript{731}NH\textsubscript{2}Y-α2) and at 8.65 (for Y\textsubscript{730}NH\textsubscript{2}Y-α2) are shown in Fig. S8. The kinetic parameters are summarized in Table 1.

Fits to the kinetic traces in all cases required three exponentials. These results contrast to those with wt-β2/NH\textsubscript{2}Y-α2, which generate a NH\textsubscript{2}Y• with all substrate/effectator pairs in two kinetic phases. The molecular basis for the different phases is not understood, but our interpretation is that the slowest kinetic phase is associated with dCDP formation as observed in the steady state pH rate profile studies with F\textsubscript{3}Y\textsubscript{356-β2/α2} and CDP/ATP (18). The second, kinetic phase may be associated with the mutations (V\textsubscript{353}G and S\textsubscript{354}C) that, as noted above, are required for efficient semisynthesis of β2. We propose that these two residues are likely located at the α2/β2 interface generating an additional conformation that binds to α2 and results in NH\textsubscript{2}Y• formation. We propose that the fastest phase is associated with non-productive conformational changes, as noted above. Three kinetic phases have previously been observed when fitting the data associated with 3-hydroxytyrosine radical (DOPA•) formation from DOPA-β2/α2/CDP/ATP, also made by the EPL method (17). The analysis of these pH profiles presented below focuses mainly on the slowest, third kinetic phase, as it corresponds to dCDP production in the steady state.

**Analysis of the pH rate profile for NH\textsubscript{2}Y• formation with F\textsubscript{3}Y\textsubscript{356-β2/NH\textsubscript{2}Y-α2**

The pH dependence of wt RNR activity is not understood, but is associated with conformational changes and not chemistry. In the current case, pH is used to modulate the driving force for radical transfer (Figs. 1C & 1D) based on our previously observed correlation between the rate of dNDP formation and the E\textsubscript{p} difference between F\textsubscript{3}Y and Y between pH 7.8 and 8.7. To analyze the results in Fig. 3, the rate constants for NH\textsubscript{2}Y• formation were plotted as a function of pH for each kinetic phase (Figs. 4A–4C).

The data from the fastest phase with NH\textsubscript{2}Y\textsubscript{730-α2} (Fig. 4A, blue dots) and NH\textsubscript{2}Y\textsubscript{731-α2} (Fig. 4A, red dots) exhibited k\textsubscript{obs} that vary from 2.7–48.9 s\textsuperscript{-1} and from 7.8–26.2 s\textsuperscript{-1}, respectively. The amplitudes in this phase account for the smallest amount, 10–26 %, of the total NH\textsubscript{2}Y•. Furthermore, unlike the other phases, the profile for NH\textsubscript{2}Y\textsubscript{731-02} is distinct from NH\textsubscript{2}Y\textsubscript{730-α2} (Figs. 4A and S9). The second kinetic phase exhibits rate constants from 0.38–3.8 s\textsuperscript{-1} (0.80–3.0 s\textsuperscript{-1}) with amplitudes of 37–50 % of the total absorbance change (Fig. 4B, blue dots and red dots), while the k\textsubscript{obs} for the third kinetic phase vary from 0.11–0.52 s\textsuperscript{-1} (0.09–0.42 s\textsuperscript{-1}) with amplitudes of 27–52 % of the overall change (Fig. 4C, blue dots and red dots). The profiles of the two slow kinetic phases have shapes very similar to that observed for dCDP formation with F\textsubscript{3}Y-β2.

Our hypothesis is that the slowest kinetic phase is associated with the RNR conformation active in turnover. To analyze this phase further, the rate constants for NH\textsubscript{2}Y• formation with both 730 and 731 mutants were overlaid with those for dCDP formation in the steady state (Fig. 4D). Also included is the profile for dCDP formation with intein wt-β2 (green squares). A direct comparison between the rate constants for pre-steady state NH\textsubscript{2}Y• formation and steady state dCDP formation (by F\textsubscript{3}Y\textsubscript{356-β2/α2}) as a function of pH shows remarkable agreement. They overlap in all three activity regimes and are distinct from that of intein wt-β2 at pH > 7.8 (Fig. 4D, green squares). Because NH\textsubscript{2}Y• formation serves as a read-out for forward radical transfer, the results indicate that a step in this process has the same pH-dependent rate constants as k\textsubscript{cat} measured by steady state kinetic assays. This
observation supports our original proposal that at high pH, the rate-determining step has shifted from a physical step to radical transfer and suggests that insertion of F₃Y results in a decrease in the rate constant for forward radical transfer as the reaction pH is increased.

Discussion

Kinetic Simulations

To address our inability to detect radical intermediates with F₃Yβ₂, despite an apparent shift in the rate-determining step, kinetic simulation studies have been carried out. Fig. 1D will be used as a means to predict the potential build-up of intermediates associated with F₃Y insertion into the pathway. This energy landscape model of the pathway assumes that the reduction potentials and pKₐs of the residues involved are minimally perturbed by the protein environment. For residues Y₃₅₆ in β2 and Y₇₃₀ and Y₇₃₁ in α₂, these assumptions are supported by our recent studies in which 3-nitrotyrosine (NO₂Y) has been site specifically incorporated into each of these positions (44,45). The model suggests that during forward PCET, the step most likely to be rate-limiting at pH 8.4 would be the oxidation of F₃Y by the W₄₈• based on Eₚs of 0.85 V and 0.8 V, respectively, (Figs. 1C & 1D). In the reverse direction at pH 8.4, the slowest step would be the oxidation of F₃Y by Y₇₃₁• consistent with Eₚs of 0.85 V and 0.74 V, respectively (Figs. 1C & 1D).

The kinetic model in Fig. 5 is based on our previous kinetic model for wt RNR at pH 7.6 and the data and simulations reported herein (Fig. 5) (36). Two points should be reiterated prior to presentation of the model in detail. The first is that intein-wt β2 has 25 % the activity of wt-β2 due to the two additional mutations. Thus, the rate constants associated with the conformational change(s) used in the model could be elevated 4-fold for the wt RNR. The second is that while W₄₈ has been incorporated into the original pathway model (13) and all subsequent renditions of this model (15,24,46), there is currently no direct evidence for its involvement in contrast with the proposed Y pathway residues. While in our model we have incorporated this residue, we and others are actively trying to address its involvement using multiple methods (45,46).

In the model in Fig. 5, binding of CDP and effector ATP, step A, is followed by the rate-limiting conformational change, step B, that gates radical transfer. Subsequent to this change, Y₁₂₂• is reduced and gives rise to a W₄₈•, step C, which then generates a C₄₃₉• via F₃Y₃₅₆, Y₇₃₁ and Y₇₃₀ transient radical intermediates, step D. One hopping step within step D, oxidation of F₃Y by W₄₈•, is proposed to be the rate-limiting step in the forward direction. C₄₃₉• catalyzes nucleotide reduction, step E, which then gives rise to Y₇₃₁• through a transient Y₇₃₀• intermediate, step F. Y₇₃₁• then regenerates Y₁₂₂• via F₃Y₃₅₆ and W₄₈•, step G; this step could represent the rate-limiting step in reverse radical transfer. Dissociation of dCDP, step H, completes the catalytic cycle, although in the steady state the active site disulfide needs to be re-reduced for multiple turnovers to occur. In this model, the Kᵩs and rate constants in black (steps A, E, F and H) have been determined experimentally (45,47,48). A rate constant of 107 s⁻¹ for dCDP formation, step E, and 100 s⁻¹ for Y₃₅₆• formation, step F, have been recently determined in studies using NO₂Y site-specifically incorporated in place of Y₁₂₂ (NO₂Y₁₂₂-β₂) (45). This protein does not have the additional mutations of the EPL-generated protein. A NO₂Y• is transiently generated at position 122, that uncouples proton and electron transfer and unmasks for the first time, the rate constant of dCDP formation and for Y₃₅₆• formation in the reverse direction. The rate constants in grey (steps C, F and kₑv in step B) were used in our original kinetic model for wt-RNR (pH 7.6) to reproduce our inability to observe disappearance and reappearance of Y₁₂₂• or to detect any radical intermediates under a wide range of conditions (36). The rate constants in red (kₑv in steps B, D, and G) have been determined experimentally herein and assigned to these steps as described subsequently. Note that the rate constant associated with step B (0.5
s\(^{-1}\)) might be elevated 4-fold as described above. The rate constants in blue have been simulated herein.

We began this exercise by simulating the effect of a solely rate-limiting reverse radical transfer. In this case, step D and the intermediate preceding it were eliminated from the model, and we assigned to step G the \(k_{\text{obs}}\) of 0.2 s\(^{-1}\), obtained at pH 8.4 by SF absorption spectroscopy for NH\(_2\)Y• formation (see Table 1). This assignment is consistent with \(E_p\) differences between F\(_3\)Y and Y at pH 8.4 described above (25). The simulations show that with 20 \(\mu\)M RNR, Y\(_{731}\) would build up to > 12.5–14 \(\mu\)M. The RFQ-EPR studies, however, failed to detect any new Y•. Changing the parameters for step C (100–300 s\(^{-1}\) for \(k_{\text{rev}}\)) or step G (1-100 s\(^{-1}\) for \(k_{\text{rev}}\)) still yielded >12 \(\mu\)M of Y\(_{731}\)•. Thus, within the current kinetic framework, and the caveat that the Y• would have detectable hyperfine by EPR spectroscopy (42), reverse radical transfer is not solely rate-limiting, consistent with our SF absorption studies where NH\(_2\)Y-\(\alpha\)2 served as a reporter for forward radical transfer.

We next assumed a solely rate-limiting forward radical transfer. In this case, step G and the intermediate preceding it were eliminated from the model (Fig. 5) and the slow step of 0.2 s\(^{-1}\) at pH 8.4 (see Table 1) was applied to step D, consistent with \(E_p\) difference between W and F\(_3\)Y (Figs. 1C & 1D). The kinetic simulations show at 20 \(\mu\)M RNR, that W\(_{48}\)• accumulates to 0.5–1.4 \(\mu\)M (using 10\(^2\)–10\(^3\) s\(^{-1}\) for step F and 300 s\(^{-1}\) for \(k_{\text{rev}}\) of step C). As discussed above, these concentrations are at or below our lower limit of detection. In addition, given the half-sites reactivity for RNRs that we have observed in many of our experiments (17,19), the actual concentration of the radical would be even lower (0.25–0.7 \(\mu\)M). The \(k_{\text{rev}}\) for step C had to be increased to \(\geq\) 300 s\(^{-1}\) relative to our original kinetic model for RNR (200 s\(^{-1}\)) in order to reproduce our inability to detect intermediates. This suggests an increased flux toward Y\(_{122}\)• reformation when F\(_3\)Y replaces Y\(_{356}\)•. The faster the \(k_{\text{rev}}\) for steps B or C, the lower the amount of W\(_{48}\)• that would build up. This simulation makes it much less likely that a W\(_{48}\)H•+ is involved during forward PCET (Figs. 1C & 1D, see below).

Finally, we assumed that forward and reverse radical transfers are both partially rate-limiting and assign steps D and G both to 0.2 s\(^{-1}\). In this case, the simulations show that Y\(_{731}\)• and W\(_{48}\)• would build up to <0.8 \(\mu\)M and 0.25–0.7 \(\mu\)M (after accounting for half-sites reactivity), respectively. Thus, within the framework of the current model, rate-limiting forward or partially rate-limiting forward and reverse PCET are consistent with the inability to observe intermediates despite an apparent change in rate-limiting radical transfer as a result of F\(_3\)Y insertion into the pathway. This model serves as a starting point for examining the kinetics of F\(_3\)Y-\(\beta\)2 further, for example by using rapid chemical quench methodology to examine the rate of dCDP formation. The two models make different predictions about the lag phases in dCDP production. Evolution of a tRNA/tRNA synthetase pair for incorporation of F\(_3\)Y is in progress. These studies should remove the kinetic complexity associated with the two additional mutations required by EPL method, increase the overall activity 4 fold and make further analysis simpler.

**Implications for Radical Propagation**

Despite the shift in the rate-determining step associated with the redox properties of F\(_3\)Y relative to W\(_{48}\) or Y\(_{731}\) (Figs. 1C & 1D), no pathway intermediates are detectable. A modeling exercise incorporating recent kinetic parameters from studies with Y\(_{122}\) replaced with NO\(_2\)Y, provides insight as to why this might be the case, and if W\(_{48}\) is on the pathway, as to why a W• and not a WH•+ is the most likely intermediate between Y\(_{122}\) and residue 356. We consider the WH•+ less likely given the following argument. If we assume the \(pK_a\) of F\(_3\)Y is minimally perturbed (44) and that a WH•+ participates in the pathway, the \(E_p\) difference between WH•+ and F\(_3\)Y would not change between pH 7 and 8.4 (Fig. 1C). Our
data indicates that the rate-determining step occurs between Y$_{122}^\bullet$ reduction and NH$_2$Y oxidation. Therefore, the rate-limiting step would be associated with oxidation of W$_{48}$ by Y$_{122}^\bullet$ (oxidation of F$_3$Y by WH•+ is pH-independent and that of Y$_{731}$ by F$_3$Y• is thermodynamically favored, Fig. 1D). This conclusion is inconsistent with the observation that when F$_3$Ys are placed in position 356, their pH rate profiles differ from intein wt-β2. They would be the same if Y$_{122}^\bullet$-mediated W oxidation was slow. It is also inconsistent with the >200 mV $E_p$ difference (Fig. 1D, Y$_{122}$ is special and appears to be a thermodynamic hole) at pH 7 and a >310 mV difference at pH 8.4 between Y$_{122}$ and WH•+. With W$_{48}$• as an intermediate, on the other hand, an explanation for the change in rate-determining step when F$_3$Y is inserted into the pathway becomes apparent. In the case of the neutral W•, the $E_p$ gap between Y$_{122}$ and W$_{48}$ does not change as a function of pH, F$_3$Y•-mediated oxidation of Y or NH$_2$Y is thermodynamically very favorable (Fig. 1D), and thus oxidation of F$_3$Y by W$_{48}$• remains as the basis for the observed changes. Between pH 7 and 8.4 (Fig. 1C) the reaction becomes less favorable by 70 mV.

While our previous studies have demonstrated the importance of a WH•+ in active metallocofactor assembly and more specifically Y$_{122}$ oxidation (49,50), in this case the reaction is irreversible, in contrast to the reversible PCET pathway (Fig. 1), and the “hot” Fe$^{4+}$/Fe$^{3+}$ oxidant could drive the reaction toward the Y•. These studies and our other studies suggest that Nature has chosen W and Y as reversible redox conduits over long distances as their reduction potentials require minimal perturbation. W and Y contrast to many metal or organic cofactors (flavins, hemes) used by enzymes in which the protein environment must modulate the reduction potentials by >500 mV for the cofactor to function (51,52). W and Y have the appropriate chemical properties for fine-tuning the unusual radical propagation pathway in RNR.

The kinetic modeling provides a framework to think about optimal unnatural amino acids to perturb the pathway to detect intermediates and to study the PCET process at each step. For example, the model suggests that to detect intermediates in the pathway, a hot oxidant needs to replace the Y$_{122}^\bullet$. Rapid reduction of the hot oxidant would lead to rapid production of pathway intermediates that would be unable to reoxidize the reduced form of the oxidant, allowing build up an intermediate(s). This approach has recently been shown to be successful by placing NO$_2$Y at position 122 that can be oxidized by the Fe$^{4+}$/Fe$^{3+}$ radical, but cannot be reoxidized by pathway radicals (45). The studies further suggest that NH$_2$Y substitution will be useful in examining the individual hopping steps of the three transiently involved Y•s in the pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Ellen C. Minnhan for helpful discussions and the NIH (Grant 29595 to J.S.) for support of this work. M.R.S. is a Novartis Fellow of the Life Sciences Research Foundation.

References


**Abbreviations**

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>$\alpha$</td>
<td>ribonucleotide reductase large subunit</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>$\beta$</td>
<td>ribonucleotide reductase small subunit</td>
</tr>
<tr>
<td>C•</td>
<td>thyl radical</td>
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<tr>
<td>CDP</td>
<td>cytidine-5’-diphosphate</td>
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<tr>
<td>DOPA</td>
<td>3,4-dihydroxyphenylalanine (or 3-hydroxytyrosine)</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
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<td>EPL</td>
<td>expressed protein ligation</td>
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<td>electron paramagnetic resonance</td>
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<tr>
<td>NDP</td>
<td>nucleoside 5′-diphosphate</td>
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<td>NH2Y</td>
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<td>PCET</td>
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Figure 1.
The proposed radical initiation pathway and its energetics in E. coli RNR with site-specific incorporation of unnatural amino acids (13). Residues in grey are associated with the diferric cluster, in black are proposed pathway residues (13), and in blue are the unnatural amino acids (F₃Y and NH₂Y) to probe the pathway in the present studies. Note that the structural location of Y₁₂₂ is unknown. (C) Peak potentials (Eₚ) for free (NH₂Y), and N-acetylated and C-amidated (Y, W, F₃Y) amino acids, as a function of pH. The Eₚs for Y•, W• and 2,3,5-F₃Y• have been previously determined (25) and the trace for NH₂Y• has been generated from the reduction potential determined at pH 7, assuming Nernstian behavior (27). (D) The Eₚs from panel (C) have been assigned to residues in the radical propagation pathway to provide a qualitative energy landscape. Red and blue rectangles represent the peak potentials for each amino acid at pH 7.0 and 8.4, respectively. The peak potentials of WH•+ and F₃Y• are represented by red rectangles with blue diagonal lines as they do not change between pH 7–8.4. The Eₚ range for Y₁₂₂• is expanded (indicated by shading), because its properties relative to the other three Ys, including its pKₐ, are unique. Y₁₂₂• has a half-life of ~4 days and likely represents a thermodynamic hole (45). Brackets are placed around W₄₈ as no direct evidence is available that places it on the pathway. If it is on the pathway, its protonation state, W₄₈H•+ vs. W₄₈•, that participates in radical transfer is unknown, and therefore Eₚs for both of these species are included.
Figure 2.
SF absorption and RFQ EPR spectroscopies with F3Y356β2 in the presence of α2 and CDP/ATP at pH 8.4. (A) Single λ SF time courses monitored at 410 nm (black), 510 nm (red) and 560 nm (blue). (B) RFQ EPR spectra of samples quenched at 72 ms (black), 138 ms (blue) and 1.9 s (red) with the EPR spectrum recorded at 77 K. The EPR spectrum of the 1.9 s quench time point was collected at 15 K (green).
Figure 3.
SF absorption spectroscopy of NH$_2$Y-$\alpha$2s/F$_3$Y$_{356}$-$\beta$2/CDP/ATP as a function of pH. Reaction of F$_3$Y$_{356}$-$\beta$2 with NH$_2$Y$_{730}$-$\alpha$2 (A) or NH$_2$Y$_{731}$-$\alpha$2 (B). Each trace is an average of 6–8 traces. Black lines describe tri-exponential fits to the data. See Table 1 for kinetic parameters.
Figure 4.
pH rate profiles for NH$_2$Y$^*$ formation in the reactions of NH$_2$Y$_{730}$-α2 (blue dots) or NH$_2$Y$_{731}$-α2 (red dots) with F$_3$Y$_{356}$-β2 in the presence of CDP/ATP. pH dependence of the rate constants from the 1st (A), 2nd (B) and 3rd (C) kinetic phases determined from the tri-exponential fits (Table 1). In (D), the data from panel (C) are overlaid with the pH rate profiles for [${}^{14}$C]-dCDP formation with F$_3$Y$_{356}$-β2/wt α2 (black dots) and with intein wt-β2/wt α2 (green squares), determined in a previous study (18).
Figure 5.
Kinetic model for the catalytic cycle of F3Y356β2 with wt α2 and CDP/ATP, which have been omitted for clarity. The $K_d$s and the rate constants in black have been measured experimentally (45,47,48), the rate constants in grey have been adapted from our previous simulations (36) and those in red have been measured herein and assigned to steps B, D, and G as described in the text. Rate constants in blue have been simulated herein. When assuming rate-limiting reverse PCET, step D and the intermediate prior to D were eliminated from the model, and the $k_{obs}$ of 0.2 s$^{-1}$ was assigned to step G. When assuming a rate-limiting forward PCET, step G and the intermediate prior to G were eliminated from the model and the $k_{obs}$ of 0.2 s$^{-1}$ was assigned to step D. The $k_{rev}$ in steps C, D and G have been assigned 200-300 s$^{-1}$, 10 s$^{-1}$ and 10 s$^{-1}$, respectively, to reproduce the lack of observable intermediates (i.e. $W_{48^*}$).
Table 1

Summary of the kinetic parameters for NH\textsubscript{2}Y\textbullet formation in the reaction of NH\textsubscript{2}Y\textsubscript{730-\alpha2} or NH\textsubscript{2}Y\textsubscript{731-\alpha2} with F\textsubscript{3}Y\textsubscript{356-\beta2} and CDP/ATP.

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<th>k\textsubscript{obs} (s\textsuperscript{-1})&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Amp&lt;sup&gt;b&lt;/sup&gt; (% Y\textsubscript{122\textbullet})</th>
<th>k\textsubscript{obs} (s\textsuperscript{-1})&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Amp&lt;sup&gt;b&lt;/sup&gt; (% Y\textsubscript{122\textbullet})</th>
<th>k\textsubscript{obs} (s\textsuperscript{-1})&lt;sup&gt;a&lt;/sup&gt;</th>
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<sup>a</sup>Estimated errors based on systematic factors.

<sup>b</sup>The amount of NH\textsubscript{2}Y\textbullet trapped has been reported as a % of total initial Y\textsubscript{122\textbullet}, which in these experiments was 4.8 \textmu M.