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Conformationally-Dynamic Radical Transfer within Ribonucleotide Reductase

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Abstract

Ribonucleotide reductases (RNR) catalyze the reduction of nucleotides to deoxynucleotides through a mechanism involving an essential cysteine based thiyl radical. In the *E. coli* class 1a RNR the thiyl radical (C₄₃₉•) is a transient species generated by radical transfer (RT) from a stable diferic-tyrosyl radical cofactor located >35 Å away across the α₂:β₂ subunit interface. RT is facilitated by sequential proton-coupled electron transfer (PCET) steps along a pathway of redox active amino acids (Y₁₂₂β ↔ [W₄₈β?] ↔ Y₃₅₆β ↔ Y₇₃₁α ↔ Y₇₃₀α ↔ C₄₃₉α). The mutant R₄₁₁A(α) disrupts the H-bonding environment and conformation of Y₇₃₁, ostensibly breaking the RT pathway in α₂. However, the R₄₁₁A protein retains significant enzymatic activity, suggesting Y₇₃₁ is conformationally dynamic on the timescale of turnover. Installation of the radical trap 3-amino tyrosine (NH₂Y) by amber codon suppression at positions Y₇₃₁ or Y₇₃₀ and investigation of the NH₂Y• trapped state in the active α₂:β₂ complex by HYSCORE spectroscopy validate that the perturbed conformation of Y₇₃₁ in R₄₁₁A-α₂ is dynamic, reforming the H-bond between Y₇₃₁ and Y₇₃₀ to allow RT to propagate to Y₇₃₀. Kinetic studies facilitated by photochemical radical generation reveal that Y₇₃₁ changes conformation on the ns-μs timescale, significantly faster than the enzymatic *k*_{cat}. Furthermore, the kinetics of RT across the subunit interface were directly assessed for the first time, demonstrating conformationally dependent RT rates that increase from 0.6 to 1.6 × 10⁴ s⁻¹ when comparing wild type to R₄₁₁A-α₂, respectively. These results illustrate the role of conformational flexibility in modulating RT kinetics by targeting the PCET pathway of radical transport.

Graphical Abstract

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. Supporting Information. Materials, methods, calculations of TA kinetics and Dutton ET distance, stopped flow fitting results, EPR spectra of 3-aminotyrosine samples used for HYSCORE, HYSCORE spectra and analysis, time resolved emission traces and fitting results, steady state emission spectra, photochemical turnover experiments, 2,3,6-F₃Y₃₅₆• transient absorption spectrum and kinetics.

INTRODUCTION

Many enzymes catalyze chemical reactions that involve the movement of charge. Electron transfer (ET) between donor-acceptor pairs within the protein milieu has been well described by a tunneling model, for which ET can occur $>10 \text{ \AA}$ at rates that are competitive or exceed the requisite k_{cat} .^{1,2} While this framework describes most ET reactions well with noted exceptions,^{3,4} it does not address the coupling of an electron to a proton, as is obligatory for radical transfer (RT). In proton-coupled electron transfer (PCET),⁵ the wavefunctions of both the electronic and nuclear coordinates mix and accordingly, the very different length scales between proton and electron transfer result in a much greater sensitivity of a PCET pathway to distance as compared to an ET pathway. Proteins are soft materials and exhibit significant degrees of freedom, from global changes in quaternary structure and phonon-like collective motion⁶ to local amino acid sidechain flexibility. This conformational sampling alters charge transfer distances and whereas modest perturbations on ET rates are imposed relative to those predicted from the static structural models,¹ the more sensitive PT rates may be dramatically affected and consequently the thermodynamics and kinetics of PCET may be significantly influenced.^{7–9} Defining the conformational landscape of redox active groups involved in PCET is therefore essential to understanding function, especially in those enzymes that derive their activity from radicals.

Endogenous amino acid residues within proteins and enzymes are the sites of RT. Due to the correlation between local structure (e.g., H-bonding) and PT, the reactivity of redox active amino acids is expected to be tuned by conformational motions owing to changes in PCET distance and potentially the identity of donor/acceptor sites for PT. Few studies, however, have directly addressed the role of either global or local conformational dynamics on the PCET chemistry of redox active amino acids, particularly with regard to the PT component.

Of the large and growing number of enzymes known to be dependent on redox active amino acids,^{10–13} ribonucleotide reductase (RNR) is an enzyme that is conformationally dynamic and employs redox active amino acid residues to perform RT and redox catalysis over an exceptionally long distance. RNR catalyzes the conversion of nucleotides (CDP, GDP, UDP, and ADP) to deoxynucleotides, the bottleneck in *de novo* DNA synthesis in all living organisms.^{10,14} Nucleotide reduction is regulated at a number of levels including allosteric regulation through effector binding sites which governs both specificity and overall activity. Nucleotide reduction is initiated by H-atom abstraction from a thiyl radical located on a conserved cysteine residue.¹⁵ The class 1a RNR of *Escherichia coli* (*E. coli*) is proposed to be a heterodimer of homodimers with an $\alpha_2\beta_2$ structure based on shape complementarity of

the composite α_2 ¹⁶ and β_2 ¹⁷ X-ray structures, small angle X-ray scattering (SAXS) and electron microscopy (EM) measurements¹⁸ as well as pulsed electron-electron double resonance (PELDOR) spectroscopic studies.¹⁹ The active cysteine thiol radical in α_2 (C₄₃₉• in *E. coli* numbering scheme) is generated transiently under turnover conditions by a stable diferric-tyrosyl radical cofactor (Y₁₂₂•) located in the β_2 subunit >35 Å away.^{18,20} Several conserved aromatic amino acid residues are proposed to be involved in RT from Y₁₂₂β to C₄₃₉α, as summarized in Figure 1a (Y₁₂₂β ↔ [W₄₈β]? ↔ Y₃₅₆β ↔ Y₇₃₁α ↔ Y₇₃₀α ↔ C₄₃₉α).^{20,21} This radical hopping pathway employs sequential PCET events,^{7,21–23} and PT and ET are necessarily regulated to maintain RT fidelity, and therefore enzymatic activity.

The mechanism of PCET that enables RT has been the subject of extensive study.^{20,22,24,25} Based on the expected distance between the stable Y₁₂₂• and Y₃₅₆ in the β subunit,²⁶ as well as the involvement of a water molecule bound to the di-iron cofactor as a proton donor during transient reduction of Y₁₂₂•,²⁷ an orthogonal PCET mechanism in β has been proposed,²¹ where PT and ET occur between distinct donor/acceptor pairs.^{28,29} Conversely, direct hydrogen bonding interactions of Y₇₃₁, Y₇₃₀ and C₄₃₉, revealed by high field electron nuclear double resonance (ENDOR) spectroscopy^{30,31} has led to the contention that RT within α_2 occurs via collinear PCET where protons and electrons are shuttled between adjacent pathway residues.²¹ At present the mechanism of RT across the $\alpha_2\beta_2$ subunit interface remains unknown. Recent spectroscopic studies indicate no direct H-bond is formed between Y₃₅₆β and Y₇₃₁α suggesting orthogonal PCET with alternative proton acceptors.³² Thus, during RT, Y₇₃₁ may act as a “gating” residue that facilitates a mechanistic switch of PCET, but the origin of this switch and its potential implications remain to be established.

The mechanistic role of Y₇₃₁ is further complicated by additional studies that suggest the Y₇₃₁ conformation may be dynamic. In the X-ray structure of the 3-amino tyrosine (NH₂Y) substituted Y₇₃₀NH₂Y- α_2 , significant electron density is observed in an alternative Y₇₃₁ conformation that differs from the π - π stacked “Y-Y dyad” conformation.³³ A similar observation was reported in the structure of the α_2 subunit of the class 1b enzyme from *Salmonella typhimurium*,³⁴ although all structures to date have been solved in the absence of β_2 . In the *E. coli* class 1a RNR, the α/β interface is relatively weakly associated and thought to be solvated to some degree^{32,35} suggesting that some heterogeneity may be possible. In addition, R₄₁₁ in α_2 has been suggested to directly H-bond to Y₇₃₁ or to order a water molecule(s) that is H-bonded to Y₇₃₁,^{31,36} thus stabilizing the π - π stacked conformation of the Y–Y dyad and perturbing the collective dyad reduction potential.³⁷ When A is substituted in place of R₄₁₁, Y₇₃₁• does not form an H-bond with Y₇₃₀ and moves closer to the β subunit across the $\alpha:\beta$ interface, thereby breaking the cofacial stacking of the Y–Y dyad.³⁶ To reconcile this observation with the current model for RT by PCET, Y₇₃₁• must then “flip” back to the π - π stacked conformation, reversibly and in a kinetically competent fashion, to accept a proton and electron from Y₇₃₀ to continue RT.^{33,36} While the observation of steady state enzymatic activity would suggest as much, direct experimental evidence for such conformational dynamics affecting PCET is lacking. More generally, experimentally addressing both PCET and conformational dynamics is a challenge of significant interest in biophysical studies as static structural models may not represent the dominant PCET active conformations involved in charge transfer.^{38,39}

Probing RT dynamics in RNR are difficult to isolate because (1) the rate determining conformational change required for RT initiation in β_{40} and timescale of (PC)ET are disparate,^{41,42} and (2) RT intermediates are only fleetingly populated owing to an uphill thermodynamic landscape (>200 mV, Figure 1b).^{28,42}

We now address the role of Y₇₃₁ as a critical linchpin in the dynamic conformational gating of the PCET pathway and RT in RNR by employing two complementary methodologies. First, we install the non-native tyrosine analogue 3-amino tyrosine (NH₂Y) by amber codon suppression technology at either Y₇₃₁ or Y₇₃₀ within the R₄₁₁A background. This residue acts as a radical trap (E vs. Y < -200 mV,⁴³ Figure 1b), allowing us to monitor H-bonding interactions at the phenoxy radical site directly by hyperfine sublevel correlation (HYSCORE) spectroscopy, thus revealing the conformational changes occurring upon RT. Due to the significant thermodynamic sink imposed by the 3-NH₂Y, these mutants are inactive or nearly inactive, but remain useful for examination of pathway radicals. Second, we employ the photo β_2 methodology^{44,45} in which radicals can be initiated on the RT pathway by photochemical oxidation of pathway Ys. In this methodology, radical generation is gated by a short laser pulse with sufficient time resolution to examine RT kinetics, unmasking RT from slower conformational gating processes. The photo β_2 is furnished by covalently ligating a bromomethylpyridine rhenium tricarbonyl phenanthroline ([Re]) photo-oxidant to β_2 via a S₃₅₅C mutation that is directly adjacent to the RT pathway residue Y₃₅₆. By following [Re] emission and Y₃₅₆• decay kinetics via transient optical spectroscopy, we establish a lower bound for the rate of conformational dynamics and directly assess the rate constants for radical injection from Y₃₅₆• to Y₇₃₁•. Our results demonstrate a direct link between conformational dynamics and PCET kinetics in RNR, resulting from the structural sensitivity of PCET to its strong dependence on distance and local protein environment. Dynamics such as those reported herein may not be apparent from equilibrium measurements, but are likely critical in many other enzymes that utilize amino acids that participate in PCET.

Results

NH₂Y labelled α_2 proteins were produced by amber codon suppression methods.⁴⁶ Photo β_2 s were produced as described previously^{41,45} and installation of 2,3,6-F₃Y at position 356 was accomplished by amber codon suppression as described previously.³⁵ Detailed methods are provided in the Materials and Methods section of the SI. With labelled proteins in hand, the following experiments were performed where the R₄₁₁A- α_2 proteins are compared to the otherwise “native” α_2 proteins without the R₄₁₁A mutation. For the sake of clarity, we refer to the double mutant protein constructs containing the R₄₁₁A mutation with a prime (i.e. Y₇₃₁F vs. Y₇₃₁F' (Y₇₃₁F with R₄₁₁A mutation); Y₇₃₀NH₂Y vs. Y₇₃₀NH₂Y' (containing the R₄₁₁A mutation).

Radical Transfer Quantitation

X-band electron paramagnetic resonance (EPR) spectroscopy was performed to quantitate RT from Y₁₂₂• to the Y_{731/730}NH₂Y• radical trap in the presence and absence of the R₄₁₁A mutation. Mixing of WT- β_2 (1.2 Y•/ β_2) with Y₇₃₀NH₂Y- α_2 , CDP (substrate) and ATP

(allosteric effector) followed by freeze quenching in liquid N₂ by hand after 10 seconds resulted in 48% conversion of Y₁₂₂• signal to Y₇₃₀NH₂Y•, as determined by subtraction of the Y₇₃₀NH₂Y• spectrum from the composite spectrum (52% Y₁₂₂•) and double integration of each component (Figure S1), similar to previous reports.^{46,47} These values represent nearly stoichiometric RT (excluding 5–10% total spin loss determined from small scale EPR quantitation similarly as previously described⁴⁷) as the active $\alpha_2\beta_2$ complex exhibits “half-site reactivity” (Figure S1 schematic), where only one of the two Y₁₂₂• of each β_2 is translocated onto the RT pathway at a time.^{19,48,49} The X-band EPR experiment was repeated for Y₇₃₀NH₂Y'– α_2 , Y₇₃₁NH₂Y– α_2 and Y₇₃₁NH₂Y'– α_2 ; significant RT to the NH₂Y trap site (>25% spin transfer) and minimal spin loss (Figure S2) was observed for all experiments.

Hydrogen Bond Environment of Radical Trap

HYSCORE spectroscopy (Figures 2 and S3–S6) of Y_{731/730}NH₂Y• trap states was employed to examine the perturbation of the H-bond structure of the radical by the R₄₁₁A mutation. ENDOR spectra for Y₇₃₁NH₂Y•, Y₇₃₀NH₂Y• and Y₇₃₁NH₂Y'• have been previously recorded^{31,36} and the corresponding HYSCORE spectra in Figures 2A, 2B and 2C, respectively, are consonant with these ENDOR results. The ENDOR data, in conjunction with DFT modelling, revealed the number of H-bonds at each respective NH₂Y site and the associated hyperfine coupling (HFC) constants consistent with the collinear PCET pathway of α_2 . The HYSCORE method provides a direct experimental measure of the A_x HFC by linear extrapolation without requiring DFT modelling. Additionally, the HYSCORE data provide an enhanced resolution of the electron-nuclear interactions of NH₂Y• with its H-bonds by spreading the spectrum out into 2D. The HYSCORE spectrum shown in Figure 2D reveals the NH₂Y environment of Y₇₃₀NH₂Y', which has not been measured by ENDOR; we include the HYSCORE results for Figures 2A–2C for completeness and for comparison to 2D.

Figures 2Ai and 2Bi (see panel ii for expanded view of dashed region of panel i) show the ¹H HYSCORE measurements on Y₇₃₁NH₂Y• and Y₇₃₁NH₂Y'•. The disappearance of the cross-ridge feature (red dashed elliptical region) in Figure 2Bii indicates the loss of an H-bond with Y₇₃₀ in the R₄₁₁A variant. The HFC constants determined from an analysis of the HYSCORE spectra in Figure 2Ai via linear regression in squared-frequency units (Figures S3–S6) give A_x = 10.4 MHz and A_{y/z} = –8.3 MHz, which are in excellent agreement with the previously reported ²H ENDOR parameters for the H-bond from the Y₇₃₀ hydroxyl (10.0 MHz and –8.5 MHz, respectively, after correcting for the gyromagnetic ratio of ¹H/²H = 6.51).³¹ ²H ENDOR also demonstrated the loss of this H-bond in the Y₇₃₁NH₂Y'• trapped state, reproduced in Figure 2B (see Fig. 2Aiii and 2Biii for schematic representation). The similarity of the results between HYSCORE and ENDOR experiments reinforce the HYSCORE method as a complementary tool for examining the H-bonding environment of NH₂Y radical trapped species.

The HYSCORE spectrum of Y₇₃₀NH₂Y• shown in Figure 2C reveals four distinct H-bonding interactions (Table S1): the 3-amino protons analogous to the Y₇₃₁NH₂Y (black arrow), the hydroxyl group of Y₇₃₁ (red arrow, HFC: A_x = 5.4 MHz and A_{y/z} = –7.8 MHz),

the thiol of C₄₃₉ (green arrow, HFC: $A_X = 1.4$ MHz and $A_{Y/Z} = -7.0$ MHz), and a weaker hydrogen bond previously assigned to water (blue arrow, HFC: $A_X = 3.5$ MHz and $A_{Y/Z} = -2.3$ MHz). These HFCs again agree well with the values reported by ²H ENDOR (Y₇₃₁-OH: $A_X = 4.6$ MHz and $A_{Y/Z} = -7.8$ MHz, C₄₃₉-SH: $A_X = 3.9$ MHz and $A_{Y/Z} = -6.7$ MHz, and H₂O: $A_X = 2.7$ MHz and $A_{Y/Z} = -1.4$ MHz). In contrast to the previously published ENDOR data, all four H-bonds are clearly resolved without the need for modelling. The fact that the experimentally determined HFCs match well with the reported ENDOR results further validates those prior studies and the DFT model. Interestingly, the spectrum of Y₇₃₀NH₂Y'• in Figure 2C is essentially identical to that of Figure 2D, with nearly identical HFC parameters (Table S1). These data suggest that radical propagation to Y₇₃₀ is associated with conformational changes in Y₇₃₁ in R₄₁₁A- α_2 , which re-establish the H-bonding interaction within the Y-Y dyad.

Radical Transfer Measurements

To examine the effect of the R₄₁₁A mutation on Y₁₂₂• reduction and NH₂Y• formation, we performed stopped flow UV-vis absorption, following mixing of Y₇₃₀NH₂Y(•) with WT- β_2 . Figure 3 shows the UV-vis traces of the stopped flow experiments where Y₇₃₀NH₂Y and Y₇₃₀NH₂Y' labelled α_2 are mixed with WT- β_2 in the presence of CDP and ATP. The optical signature of NH₂Y• ($\lambda_{\max} = 320$ nm) is distinct from native (Y₁₂₂•) or adventitiously generated Y radical ($\lambda_{\max} = 410$ nm).^{36,46} In the stopped flow experiments, mixing of Y₇₃₀NH₂Y- α_2 or Y₇₃₀NH₂Y'- α_2 resulted in biphasic transfer of the radical species from Y₁₂₂(β) to Y₇₃₀NH₂Y(α) with rate constants analogous to previous measurements.^{33,36} Fits of the data for Y₇₃₀NH₂Y• formation in Y₇₃₀NH₂Y- α_2 furnished apparent rate constants of 7.4(5) and 0.63(6) s⁻¹, representing 66% and 34% of the amplitude change respectively. The fast phase is similar to the overall steady state activity of 3–8 s⁻¹ for WT- α_2 and to prior measurements for this rate constant.^{33,50} The apparent rate constants for Y₇₃₀NH₂Y'• formation were quite similar to the single mutant at 6.2(7) and 0.30(2) s⁻¹. In contrast to the single mutant Y₇₃₀NH₂Y, the relative amplitudes of the two phases were 46% and 54%, respectively, for Y₇₃₀NH₂Y'. The origin of biphasic RT kinetics is at present unclear, and therefore the difference in the relative amplitude of the two kinetic phases is not immediately obvious. Both the rate and relative amplitude of the Y₇₃₀NH₂Y• formation in the presence or absence of the R₄₁₁A mutation are recapitulated in the Y₁₂₂• decay kinetics of proteins (Figure 3, data summarized in the Tables S2).

Radical Transfer Measurements Probed by Photo β_2 s

Photo β_2 allows for the “instantaneous” generation of radicals on the PCET pathway of RNR.^{44,45} Photoinitiated generation of Y• allows the RT kinetics to be obtained for the different assumed conformations of Y₇₃₁. In this photo β_2 , the [Re] chromophore is covalently ligated to a modified β_2 containing a S $\rightarrow$ C mutation at position 355 via a methylpyridinyl linker (SI Materials and Methods), directly adjacent to the RT pathway residue Y₃₅₆. Here, absorption of a photon by the [Re] unit excites a metal-to-ligand charge transfer transition. The excited state can directly act as an oxidant (excited state reduction potential of 1.97 V) of the adjacent Y₃₅₆. Electron transfer from Y₃₅₆ to the [Re*] can be monitored by excited state emission of the [Re*] (either by emission intensity or by excited state lifetime) with the judicious choice of a reference. To this end, we employ a Y₃₅₆F

substitution, which is redox inert, and offers a kinetic reference to interpret the subsequent emission quenching kinetics. By this methodology we have demonstrated that the [Re*] of the photo β_2 in complex with α_2 is selective for Y₃₅₆ oxidation relative to Y₇₃₁ in WT α_2 , as evidenced by the indistinguishable [Re*] decay kinetics. Given our HYSCORE data and the previously published PELDOR data, Y₇₃₁ may become susceptible to oxidation by the [Re*] excited state. Thus, the photo β_2 system may be employed for oxidation of the adjacent Y₃₅₆(β) or direct Y₇₃₁(α) oxidation with utilization of Y₃₅₆F-photo β_2 , both of which are constituents of the native PCET pathway.

Figure 4 compares the fluorescence spectrum of Y₃₅₆Fphoto β_2 in the presence of WT (blue dashed line) and Y₇₃₁F- α_2 (blue solid line). The slight decay in the fluorescence intensity from Y₇₃₁F to WT- α_2 suggests Y₇₃₁ oxidation is inefficient. Conversely, in the presence of R₄₁₁A- α_2 (green dashed line), Y₃₅₆F-photo β_2 exhibits a dramatic decrease in emission intensity relative to Y₇₃₁F'- α_2 :photo β_2 complex (green solid line), indicating significant Y₇₃₁ oxidation relative to the WT system.

Emission lifetime measurements (Figure S7 and Table 1) recapitulate the results of steady state emission measurements. Measurement of photo β_2 in the presence of Y₇₃₁F- α_2 provides the reference point for emission decay (defined by rate constant k_0) of the [Re] excited state ([Re*]), in the absence of on-pathway RT. [Re*] emission lifetimes in the presence of WT and Y₇₃₁F- α_2 are statistically identical, suggesting no radical injection directly into α_2 . For the R₄₁₁A mutants, Y₃₅₆Fphoto β_2 in the presence of Y₇₃₁F' again provides the k_0 reference for RT involving proteins with the R₄₁₁A mutation. The rate constant for RT (k_{PCET}) is obtained from the following expression,

$$k_{\text{PCET}} = \frac{1}{\tau_0} - \frac{1}{\tau_{\text{obs}}} = k_{\text{obs}} - k_0 \quad (1)$$

where k_{obs} is the decay rate constant of [Re*] in which a portion of the RT pathway is intact. Rapid RT injection ($k_{\text{PCET}} = 7.7 \times 10^5 \text{ s}^{-1}$) from [Re*] to Y₇₃₁ is observed in the R₄₁₁A variant. Furthermore, when pathway blocks Y₇₃₀F' and C₄₃₉S' were compared to Y₇₃₁F' and R₄₁₁A, a systematic increase in k_{PCET} from Y₇₃₀F' to R₄₁₁A (Figures S8 and S9 and Table 1) is observed. The increase in the quenching rate constant as the pathway is extended suggests that the radical generated on Y₇₃₁ is in rapid equilibrium along the RT pathway on the timescale of the [Re*] lifetime. That the radical is delocalized along a pathway under rapid conformational dynamics is supported by the observation of clear dCDP production over the background when the Y₃₅₆F-photo β_2 was illuminated in the presence of CDP, ATP and R₄₁₁A- α_2 (α_2 with only R₄₁₁A single point mutation) *but not* Y₇₃₁F' (Figure S10).

The F-block at Y₃₅₆ shows that [Re*] bypasses the natural PCET pathway by direct oxidation of Y₇₃₁ in α_2 . To investigate the role of R₄₁₁ in modulating RT across the subunit interface along an intact pathway, emission lifetime measurements were repeated by utilizing 2,3,6-F₃Y₃₅₆-photo β_2 (Figure 1B). 2,3,6-F₃Y₃₅₆-photo β_2 was used as a proxy for Y₃₅₆-photo β_2 because it offers the distinct advantages of: (1) being more efficiently oxidized photochemically since it remains deprotonated under the experimental conditions ($pK_a =$

7);³⁵ (2) being a stronger oxidant (by 180 mV with respect to Y at pH 8.2,⁵¹) resulting in higher efficiency radical injection into α_2 ; and, (3) displaying a significantly shifted phenoxy radical absorption peak, which can be probed selectively within the native tyrosine background (*vide infra*) while minimally perturbing the protein structure.^{51–53} [Re]* lifetime measurements were performed for 2,3,6-F₃Y₃₅₆-photo β_2 in the presence of Y₇₃₁F, WT, Y₇₃₁F', Y₇₃₀F', C₄₃₉S' and R₄₁₁A- α_2 and the results for which are displayed in Figure S11 together with the lifetimes summarized in Table S3. Clear emission quenching is observed when comparing Y₇₃₁F or R₄₁₁A:Y₇₃₁F to WT or R₄₁₁A, respectively, but the results indicate that the delocalization occurs from the 2,3,6-F₃Y• across the α_2 : β_2 protein interface. Taken together with the Y₃₅₆F-photo β_2 emission lifetime measurements, these data demonstrate that radical equilibration occurs among the Y₃₅₆ β -Y₇₃₁ α -Y₇₃₀ α RT pathway against the R₄₁₁A background on a timescale which competes with [Re*] radiative decay. Abstracting direct information about the kinetics of RT event across the α_2 : β_2 subunit interface is not possible with this data alone due to the kinetic complexity of the coupled processes, namely radical equilibration, quenching and substrate activation.

Transient Absorption Kinetics of RT

To address the role of the Y₇₃₁ conformational landscape on RT across the α_2 : β_2 subunit interface, transient absorption (TA) experiments were performed to examine directly the photo-generated 2,3,6-F₃Y₃₅₆• and its subsequent decay kinetics. As noted above, 2,3,6-F₃Y• tyrosyl radical spectrum is red shifted ~10 nm from that observed from Y• (Figure S12) allowing the kinetics of 2,3,6-F₃Y₃₅₆ to be monitored independently from on and/or off pathway Y• species. Because charge recombination with the oxidized [Re^{II}] photosensitizer precludes build-up of an observable transient signal, we employed oxidative quenching (flash-quenching) by addition of Ru^{III}(NH₃)₆ to extend the lifetime of the [Re^{II}] oxidant and prevent back ET.⁴⁴ Transient absorption (TA) spectra recorded 1 μ s after excitation of 2,3,6-F₃Y• alone and in the presence of WT, R₄₁₁A- α_2 and Y₇₃₁F'- α_2 are shown in Figure S13. In all cases, a 2,3,6-F₃Y• spectrum is observed, corresponding to 4% quantum yield. Comparison of 2,3,6-F₃Y• spectra from free 2,3,6-F₃Y₃₅₆ photo β_2 and in complex with the various α_2 reveals a shift in the absorption spectrum of the radical, consistent with the emission spectrum of [Re] and anticipated electrostatic perturbations, where solvent exclusion by complexation with α_2 as well as elimination of charge in the R₄₁₁A mutants result in blue shifts of the 2,3,6-F₃Y• resonance (Figure S13). Clear interpretation of the TA spectra to discern the kinetics for RT across the α_2 : β_2 interface are precluded by the complexity of the RT process, which stems from the nearly isopotential and reversible RT pathway within α_2 (see SI). The use of NH₂Y as a radical sink that greatly simplifies the kinetic description of the system, affording direct quantitation of k_{PCET} across the subunit interface (*vide infra*).

The TA kinetics of the 2,3,6-F₃Y• were measured in the presence of Y₇₃₁F- α_2 and the lifetime decay of 14.7(7) μ s yielded $k_0 = 6.8(3) \times 10^4 \text{ s}^{-1}$ (Table 2 and Figure S14), which provides a reference for the decay (defined by rate constant k_0) of the radical in the absence of on-pathway RT. In the presence of Y₇₃₀NH₂Y- α_2 , the 2,3,6-F₃Y• decays with a lifetime of 13.5(2) μ s yielding $k_{\text{obs}} = 7.4(1) \times 10^4 \text{ s}^{-1}$. Substitution of these two rate constants into eq (1) yields the rate constant for interfacial RT of $k_{\text{PCET}} = 6(3) \times 10^3 \text{ s}^{-1}$, which is the first

measured rate constant for Y–Y radical exchange within a protein environment and across a protein-protein interface. The 2,3,6-F₃Y₃₅₆• decay was also measured for Y₇₃₁F' and Y₇₃₀NH₂Y' (Figure 5), yielding lifetimes of 17.9(6) and 13.9(4) μs corresponding to rate constants of $5.6(2) \times 10^4 \text{ s}^{-1}$ and $7.2(2) \times 10^4 \text{ s}^{-1}$, respectively. Again, solving for k_{PCET} according to eq (1) yields $k_{\text{PCET}} = 1.6(3) \times 10^4 \text{ s}^{-1}$, which is modestly faster than the WT system and consistent with the R₄₁₁A mutation facilitating RT across the interface.

Discussion

The role of conformational dynamics in PT and ET is largely unaddressed, though often essential to describe accurately protein or enzyme function.^{9,40,54–56} Conformation is particularly pivotal to PCET as PT is acutely sensitive to small changes in donor-acceptor distance, which can be modulated by conformational dynamics. Indeed, the RT pathway of RNR is finely tuned to control PCET. The single point mutation R₄₁₁A in α₂ has been demonstrated to affect the conformation of a critical RT pathway residue, Y₇₃₁(α), breaking the π-π stacking of the Y₇₃₁–Y₇₃₀ dyad, which is required for efficient collinear PCET.³⁶ R₄₁₁A-α₂ maintains nucleotide reductase activity with a specific activity of 470(20) nmol min⁻¹ mg⁻¹ (1.3 s⁻¹), 17% that of WT enzyme.³⁶ The retained enzymatic activity suggests that the conformation of Y₇₃₁ is dynamic during RT, providing an impetus to address directly the conformational flexibility of Y₇₃₁ during RT and how conformational flexibility affects PCET kinetics across the α₂:β₂ protein interface.

The H-bonding environment of trapped NH₂Y• radicals has been shown by high field electron spin resonance spectroscopy to report on the structural consequences of conformational perturbations.^{30,31,36} ENDOR data, in concert with PELDOR measurements have probed the radical environment at positions Y₇₃₀ and Y₇₃₁ in the native enzyme,^{19,30,31} and the perturbed conformation of Y₇₃₁ in the R₄₁₁A mutant (Figure 1a).³⁶ The HYSCORE experiments reported here are complementary to ENDOR. In HYSCORE, hydrogen bonds appear as cross-ridges in a 2D correlation pattern resulting from electron-nuclear spin dipolar interactions. Tyrosyl radicals in both photosystem II and bovine liver catalase have been probed successfully by HYSCORE demonstrating that this technique is well suited to examine NH₂Y• trap states in RNR.⁵⁷

The HYSCORE spectrum of Y₇₃₁NH₂Y• shown in Figure 2A reproduces the ENDOR observation of an H-bond from Y₇₃₀ with similar HFCs. The absence of the cross-ridge pattern in the Y₇₃₁NH₂Y'• spectrum shown in Figure 2B (indicated by red ellipse) is consistent with a loss of H-bonding between Y₇₃₁• and Y₇₃₀, as has also been observed by ENDOR.³⁶ The PELDOR measurements of the Y₇₃₁NH₂Y•(α)-Y₁₂₂•(β) distance establish that the loss of this H-bonding is due to a conformational change induced by the R₄₁₁A mutation in which Y₇₃₁ “flips-out” to the α₂:β₂ interface, as shown schematically in Figure 2Biii.³⁶ The flipped-out conformation of Y₇₃₁ is manifested in the steady-state emission spectra of Figure 4 and lifetimes listed in Table 1. In the flipped-out conformation imposed by the absence of the guanidine moiety of R₄₁₁, Y₇₃₁ is more susceptible to photochemical oxidation by proximity to the [Re] unit of the photoβ₂. Accordingly [Re*] emission is quenched to a greater extent in R₄₁₁A.

Whereas the ENDOR spectrum of $Y_{730}NH_2Y^\bullet$ has been reported, and is reproduced by the HYSCORE results in Figure 2C, the hydrogen-bonding environment of $Y_{730}NH_2Y'^\bullet$ has not been measured. The striking result for $Y_{730}NH_2Y^\bullet$ and $Y_{730}NH_2Y'^\bullet$ (Figure 2D) reported here is that their HYSCORE spectra are essentially identical. These results suggest that π - π stacking of the radical in the Y-Y dyad is reformed when the radical advances to position 730 from 731 and that the H-bond between OH phenol groups flips from $Y_{731} \leftarrow Y_{730}$ (Figure 2Aiii) to $Y_{731} \rightarrow Y_{730}$ (Figure 2Ciii) and is maintained when the $R_{411}A$ mutation is present (Figure 2Diii). This interconversion of conformations for the forward propagation of the radical is consistent with the π - π stacking enabling collinear PCET and is dynamic in nature as $R_{411}A$ exhibits turnover for nucleotide reduction.

Stopped flow kinetics of $Y_{122}^\bullet$ reduction and $NH_2Y^\bullet$ formation upon mixing WT- β_2 and $Y_{730}NH_2Y$ or $Y_{730}NH_2Y' - \alpha_2$ (Figure 3) show no difference in the rate constants of RT to $Y_{730}NH_2Y$. When juxtaposed against the distinct conformational environments of Y_{731} observed by HYSCORE, the lack of significant temporal differences in RT from $Y_{122}^\bullet(\beta)$ to $Y_{730}NH_2Y(\alpha)$ or $Y_{730}NH_2Y'(\alpha)$ in stopped-flow experiments (Figure 3, Table S2) suggests that the Y_{731} conformational dynamics are masked on the turnover (millisecond) time scale and therefore the more rapid kinetic method offered by photo β_2 was employed to examine the role of conformational motion directly.

Pathway radicals were generated using photo β_2 implemented in two ways; injecting a radical directly into α_2 (with a $Y_{356}F$ -photo β_2 described above), or via radical generation at Y_{356} of the RT pathway in β_2 (with 2,3,6- F_3Y_{356} -photo β_2). Whereas, photochemical generation $Y_{356}^\bullet(\beta)$ by the photo β_2 has been examined extensively and occurs on the microsecond timescale,⁵⁸ the direct generation of $Y_{731}^\bullet(\alpha)$ to bypass Y_{356} has been achieved by the introduction of [Re] at position 356.⁵⁹

Figure S8–S10 reveals direct radical injection within the $Y_{356}F$ -photo β_2 : $R_{411}A$ - α_2 complex and that it can support enzyme turnover. Consistent with the steady-state quenching experiments, transient emission decay kinetics measurements (Figure S7 and Table 1) show that the direct generation of a pathway radical from $Y_{356}F$ -photo β_2 only occurs significantly when Y_{731} assumes a flipped-out conformation. Interestingly, the k_{PCET} rates in Table 1 follow a Dutton scaling relationship¹ where $[Re^*]-Y_{731}$ distances can be calculated based on the known G and λ of the reaction in both flipped up and flipped out conformations. The distances calculated from this analysis provide an estimate of the conformational displacement of Y_{731} following flipping of $>2.2 \text{ \AA}$ (SI), a scalar distance relative to the [Re] unit, which is in accordance with distances obtained from PELDOR measurements and from the two distinct Y_{731} conformations observed in the $Y_{730}NH_2Y \alpha_2$ structure.³⁶ This result suggests that the mechanism of PCET for Y_{731} at the interface is ET/PT. We also observe that k_{PCET} increases in the series $Y_{730}F' < C_{439}S' < R_{411}A$ (Table 1, Figure S9) indicating that the hole delocalizes over the RT pathway. If it were delocalized, we would expect nearest-neighbour quenching with no dependence on where the pathway block is located. Hole delocalization among RT pathway residues beyond the flipped out Y_{731} on the timescale of the $[Re^*]$ lifetime necessitates electronic communication, which further supports rapid dynamics between Y_{731} in a flipped-out and π - π stacked conformation. Rapid tyrosine sidechain motions (ns- μ s) have been observed by NMR and supported by

computation.^{60–62} Thus, although the R₄₁₁A mutation shifts the equilibrium conformation of Y₇₃₁ towards the “flipped-out” conformation, rapid thermal conformational sampling allows RT to be propagated through α_2 , stabilized by the H-bonding interaction with Y₇₃₀•, to the active site of nucleotide reduction.

Because Y₃₅₆ is spectrally indistinct from Y₇₃₁ and Y₇₃₀, radical injection through position Y₃₅₆ is facilitated with the use of fluorotyrosines.⁵² The spectroscopically distinct 2,3,6-F₃Y₃₅₆-photo β_2 as a probe and Y₇₃₀NH₂Y as a radical sink in α_2 simplifies the kinetics of RT because a steady-state approximation may be imposed at Y₇₃₁ according to the following RT equilibrium,

In this equilibrium, the disappearance of 2,3,6-F₃Y₃₅₆• is given by,

$$\frac{d[\text{F}_3\text{Y}_{356}^{\bullet}]}{dt} = -(k_1 + k_0)[\text{F}_3\text{Y}_{356}^{\bullet}] + k_{-1}[\text{Y}_{731}^{\bullet}] \quad (2)$$

Due to the very slow rate, k^{-2} (endergonic, $E > 300$ mV, see Figure 1b), and k_3 (endergonic, $E > 300$ mV), relative to the expectedly large k_2 , the steady-state approximation for $d[\text{Y}_{731}^{\bullet}]/dt$ (see SI) reduces Eq (2) to

$$\frac{d[\text{F}_3\text{Y}_{356}^{\bullet}]}{dt} = -(k_1 + k_0)[\text{F}_3\text{Y}_{356}^{\bullet}] \quad (3)$$

which is effectively a reformulation of Eq (1), used to determine k_{PCET} . Thus, the rate of F₃Y₃₅₆• decay can be described accurately by considering only k_1 , and k_0 , whereupon knowing k_0 (determined by measuring $d[2,3,6\text{-F}_3\text{Y}_{356}^{\bullet}]/dt$ in the presence of Y₇₃₁F or Y₇₃₁F'– α_2) one can solve for k_1 directly by measuring $d[2,3,6\text{-F}_3\text{Y}_{356}^{\bullet}]/dt$ in the presence of Y₇₃₀NH₂Y or Y₇₃₀NH₂Y'– α_2 , a measure of interfacial PCET. Y₃₅₆ oxidation occurs through an orthogonal PCET mechanism likely involving PT to water at the interface. An analogous mechanism is assumed for Y₇₃₁ oxidation, and the change in conformation of Y₇₃₁ may result in different PCET behaviour related to donor-acceptor distance in orthogonal PT and ET components. Our results demonstrate that k_{PCET} from 2,3,6-F₃Y₃₅₆ β to Y₇₃₁ α is enhanced in the presence of the “flipped out” conformation engendered by the R₄₁₁A mutation. Therefore, the conformational flipping in R₄₁₁A α_2 again appears rapid, enhancing RT between Y₃₅₆ β and Y₇₃₁ α . We speculate that the modest rate enhancement afforded by the flipped out conformation is likely due to the increased proximity of the donor acceptor pair relative to WT, analogous to the rate increase of Y₇₃₁ oxidation observed in the [Re*] quenching experiments. While the R₄₁₁A mutation enhances the interfacial RT rate, this mutation also destabilizes the α_2 : β_2 protein complex. Since RT is not rate limiting, evolution appears to have favoured increasing binding affinity relative to

enhancing RT. That the rate is not significantly slower in the flipped-out conformation also implies that Y₇₃₁• rapidly changes conformation to funnel the radical towards Y₇₃₀NH₂Y and conformational interconversion is not rate limiting.

Conclusions

Probing conformational dynamics and their effects on charge transfer remains a challenge in the field of biological charge transfer. Conformational control of charge transfer is particularly critical to biological PCET owing to the sensitivity of proton transfer to distance. Photoβ₂ constructs, together with advanced spectroscopic biophysical techniques such as HYSCORE, offer a powerful tool to correlate PCET with conformational dynamics in RNR, which is an exemplar of enzymatic activity derived from PCET. Herein, we demonstrate the first observation of radical transport across the α₂β₂ subunit interface in RNR, and that local conformational dynamics of a tyrosine (Y₇₃₁ α) sidechain during RT in a mutant construct of the class 1a RNR of *E. coli* significantly affects the PCET kinetics of this interprotein radical transfer. Dynamics analogous to those demonstrated in this report may affect other biological charge transfer reactions where protein based amino acids function in PT and/or ET by modulating donor-acceptor distances or identities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ATP	adenosine triphosphate
CDP	deoxycytosine diphosphate
dCDP	deoxycytosine diphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
EM	electron microscopy
ENDOR	electron-nuclear double resonance
ET	electron transfer
EPR	electron paramagnetic resonance
F_nY	n-fluorinated tyrosine
HFC	hyperfine constant
HYSCORE	hyperfine sublevel correlation
NH₂Y	3-aminotyrosine

PCET	proton-coupled electron transfer
PELDOR	pulsed electron double resonance
PT	proton transfer
[Re]	rhenium 1,10-phenanthroline tricarbonyl, 3-bromoethylpyridine
RNR	ribonucleotide reductase
RT	radical transfer
SAXS	small angle X-ray scattering
TA	transient absorption
WT	wild type

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Figure 1.

Radical transfer (RT) pathway, PCET mechanism and thermodynamic landscape of the *E. coli* RNR. **A** | Redox active amino acids involved in RT and presently understood PCET mechanism at each site. Of note are the disordered nature of Y₃₅₆ β , and the R₄₁₁ sidechain (green) that controls the Y₇₃₁ conformation. Whereas the tyrosine and cysteine sites of the pathway have been shown to be intimately involved in RT, the participation of W₄₈ β remains an open question. **B** | Proposed thermodynamic landscape as determined by equilibrium measurements. Tyrosine analogues 2,3,6-F₃Y (red) and NH₂Y (blue) are shown with their relative estimated reduction potentials (pH = 8.2 vs. Y).

Figure 2.

X-band ^1H HYSCORE spectra of radical trapped $\text{Y}_{73x}\text{NH}_2\text{Y}\cdot\text{-}\alpha_2\text{s}$ generated by mixing with WT- β_2 , CDP and ATP. **A** | HYSCORE spectrum of $\text{Y}_{731}\text{NH}_2\text{Y}\cdot\text{-}\alpha_2$. **B** | HYSCORE spectrum of $\text{Y}_{731}\text{NH}_2\text{Y}'\cdot\text{-}\alpha_2$. Expanded view of dashed region in panel A. Red arrow indicates H-bond assigned to Y730. Dashed region highlighted for comparison. **C** | HYSCORE spectrum of $\text{Y}_{730}\text{NH}_2\text{Y}\cdot\text{-}\alpha_2$. Schematic representation of $\text{NH}_2\text{Y}_{731}\cdot$ based on HYSCORE data. Unresolved H-bonds due to either water or R_{411} are depicted in the scheme (brown, ref. 19). **D** | HYSCORE spectrum of $\text{Y}_{730}\text{NH}_2\text{Y}'\cdot\text{-}\alpha_2$. Dashed regions in panel (i) are expanded in panel ii. Arrows in panel ii indicate H-bonds depicted in the schemes of panel iii. Dashed circle in panels Aii and Bii indicate a lost H-bond between $\text{Y}_{731}\text{NH}_2\text{Y}\cdot$ and the phenolic hydroxide of Y730 from $\text{Y}_{731}\text{NH}_2\text{Y}\cdot$ to $\text{Y}_{731}\text{NH}_2\text{Y}'\cdot$.

Figure 3.

Stopped flow UV-vis kinetics of: formation of $\text{NH}_2\text{Y}\bullet$ ($\lambda_{\text{det}} = 320 \text{ nm}$) in $\text{Y}_{730}\text{NH}_2\text{Y}$ (—) and $\text{Y}_{730}\text{NH}_2\text{Y}'\text{-}\alpha_2$ (—) mixed WT- β_2 ; and decay of $\text{Y}_{122}\bullet$ ($\lambda_{\text{det}} = 410 \text{ nm}$) in $\text{Y}_{730}\text{NH}_2\text{Y}$ (—) and $\text{Y}_{730}\text{NH}_2\text{Y}'\text{-}\alpha_2$ (—) mixed WT- β_2 . Formation and decay kinetics are described by a biexponential fit (—). Inset shows expanded view of early time fits (shaded gray region). A $10 \mu\text{M}$ solution (6 mM ATP in assay buffer) of the α_2 variants were mixed with a $10 \mu\text{M}$ solution (2 mM CDP in assay buffer) of WT- β_2 . Data is the average of at least three experimental runs.

Figure 4.

Emission spectra ($\lambda_{\text{exc}} = 355 \text{ nm}$) of $Y_{356}F$ -photo β_2 in the presence of WT (blue) or $R_{411}A$ - α_2 (green) with an intact (dotted lines) or blocked ($Y_{731}F$ or $Y_{731}F'$, solid lines) RT pathway.

Figure 5.

TA kinetic traces for 2,3,6-F₃Y₃₅₆photo β_2 in complex with Y₇₃₁F' (red) and Y₇₃₀NH₂Y' (blue) with 1 mM CDP, 3 mM ATP and 10 mM Ru(NH₃)₆Cl₂ between 1–90 μs after excitation. Mono-exponential fits to the data are displayed in black.

Table 1

[Re*] emission decay rates and PCET kinetics for Y₃₅₆Fphotoβ₂ in complex with various α₂ mutants.

α ₂	τ _{obs} /ns	κ _{PCET} /10 ⁵ s ⁻¹
Y ₇₃₁ F	727 (10)	τ ₀
WT	714 (13)	0.3 (3)
Y ₇₃₁ F'	1,100 (7)	τ ₀
Y ₇₃₀ F'	648 (20)	6.3 (5)
C ₄₃₉ S'	629 (6)	6.8 (2)
R ₄₁₁ A	594 (16)	7.7 (5)

Table 2Decay kinetics of 2,3,6-F₃Y₃₅₆[•] measurements from transient absorption.

α_2	τ_{obs} (μs)	κ_{PCET} (10^4 s^{-1})
Y ₇₃₁ F	14.7(7)	k_0
Y ₇₃₀ NH ₂ Y	13.5(2)	0.6 (3)
Y ₇₃₁ F'	17.9(6)	k_0
Y ₇₃₀ NH ₂ Y'	13.9(4)	1.6 (3)