Kinetic model for reversible radical transfer in ribonucleotide reductase

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The enzyme ribonucleotide reductase (RNR), which catalyzes the reduction of ribonucleotides to deoxyribonucleotides, is vital for DNA synthesis, replication, and repair in all living organisms. Its mechanism requires long-range radical translocation over ~32 Å through two protein subunits and the intervening aqueous interface. Herein, a kinetic model is designed to describe reversible radical transfer in Escherichia coli RNR. This model is based on experimentally studied photoRNR systems that allow the photochemical injection of a radical at a specific tyrosine residue, Y356, using a photosensitizer. The radical then transfers across the interface to another tyrosine residue, Y731, and continues until it reaches a cysteine residue, C439, which is primed for catalysis. This kinetic model includes radical injection, an off-pathway sink, radical transfer between pairs of residues along the pathway, and the conformational flipping motion of Y731 at the interface. Most of the input rate constants for this kinetic model are obtained from previous experimental measurements and quantum mechanical/molecular mechanical free-energy simulations. Ranges for the rate constants corresponding to radical transfer across the interface are determined by fitting to the experimentally measured Y356 radical decay times in photoRNR systems. This kinetic model illuminates the time evolution of radical transport along the tyrosine and cysteine residues following radical injection. Further analysis identifies the individual rate constants that may be tuned to alter the timescale and probability of the injected radical reaching C439. The insights gained from this kinetic model are relevant to biochemical understanding and protein-engineering efforts with potential pharmacological implications.

Significance

All living organisms require a balance of deoxyribonucleotides in the cell to synthesize DNA. Ribonucleotide reductase (RNR) is an enzyme crucial to this process, converting ribonucleotides to deoxyribonucleotides. To initiate this reaction, RNR transfers an electron over long distances using specific amino acids as stepping stones. Detecting this moving electron is challenging, but has been achieved by activating the process with light for modified RNRs. We use experimental data and computer simulations to build a mathematical model that describes the time evolution of electron movement along the pathway. This model provides timescales of key steps in the pathway and illustrates the effects of slowing down or speeding up each step, thereby enhancing understanding of this biochemically and pharmacologically significant enzyme.
and to be collinear in the $\alpha_2$ subunit (i.e., the proton transfers with the electron to a residue along the radical transfer pathway) (7). A wealth of information about this process has been obtained through electron paramagnetic resonance (EPR) and other spectroscopic measurements (1, 18, 20–22), introduction of noncanonical amino acids with modified redox potentials (11, 16, 17, 23), and RNRs with ligated photosensitizers (24–27).

Kinetic and thermodynamic data on the radical transfer pathway in wild-type (WT) RNRs are sparse due to rate-limiting conformational changes and the transient nature of the tyrosine radical. Estimates for the enzyme turnover rate depend on biochemical assay conditions (28), but are typically 2 to 10 s$^{-1}$, whereas the radical translocation is thought to be faster (29, 30). Thus, examining the pathway kinetics experimentally requires decoupling of the conformational gating from the radical transfer process, as well as the ability to identify the tyrosine radical at a given position of the pathway to investigate the individual PCET steps. To address these challenges, the predominant measurements of RNR kinetics have been performed with covalently attached photosensitizers and noncanonical tyrosines in positions along the pathway.

In particular, the photoRNR systems were designed to generate the radical in the middle of the pathway, allowing the study of PCET in the absence of the conformational gating that masks the PCET kinetics in typical biochemical assays. In these systems, a photosensitizer is ligated to a cysteine residue adjacent to Y356, and the resting tyrosine radical on Y122 is reduced prior to the experiment with the addition of hydroxyurea (31–34). In this case, the only way to inject a radical into the pathway and initiate turnover is by illuminating the photoRNR complex with light. As shown in Fig. 1C, the rhenium photosensitizer is attached at position Y356, and photoexcitation yields [Re$^*$], which oxidizes the neighboring Y356 (i.e., removes an electron from this tyrosine), producing a radical at this position. A flash quencher can be used to prevent charge recombination between the photosensitizer and Y356 (24). These constructs allow temporal control of radical injection, and the photogenerated radical intermediate at Y356 can be observed with transient absorption spectroscopy. With the Y122 residue blocked through prior reduction, these constructs allow the investigation of the PCET kinetics across the $\alpha/\beta$ interface and within the $\alpha$ subunit. As only the radical lifetime of Y356 can be tracked, however, the underlying kinetics of the individual steps in the $\alpha$ subunit cannot be resolved through these experiments.

In this paper, we incorporate the existing experimental data and recently obtained simulation data to construct a kinetic model for the reversible radical transfer in the $\alpha$ subunit of E. coli RNR. Such kinetic models have been utilized to understand related phenomena in other biochemical systems. Examples include modeling of electron transfer between multiple sites over different pathways in proteins (35–38) or modeling of ion and small-molecule transport that often involves conformational changes in proteins (39, 40). In particular, van Wonderen et al. (38) combined kinetic modeling and molecular dynamics simulations with pump-probe spectroscopy to determine heme-to-heme electron transfer rates for a decaheme cytochrome. In our kinetic model, the radical transfer process...
in the photoRNR systems is described with a master equation that includes the individual radical transfer steps, as well as the conformational fluctuation of Y731 at the interface between the α and β subunits. This equation is solved to determine sets of rate constants that are consistent with the Y356 radical lifetime obtained in the photoRNR experiments. Moreover, solution of this equation reveals the time evolution of radical flow along the residues of the pathway.

The aim of this work is both to present this kinetic model as a tool that can be used for future studies as more experimental data become available and to provide mechanistic and kinetic insights based on existing experimental data. Specifically, we use the kinetic model to determine ranges of rate constants for the individual steps that are masked by slower conformational changes in the WT RNR system and are not resolved in the photoRNR experimental measurements of the overall Y356 radical decay. We also use this model to predict the impact of altering individual rate constants on the timescale and probability of radical transfer from Y356 in the β subunit to C439 in the α subunit. Analysis of the time evolution of radical flow along the residues of this pathway provides mechanistic insights that are not currently experimentally accessible and illuminates strategies for controlling this process. Furthermore, this kinetic model provides the foundation for future investigations as the field advances.

**Results and Discussion**

**Model Construction.** The kinetic model for radical transfer in photoRNR was constructed to mimic the experiments as closely as possible. This process can be viewed as irreversible radical injection, where the radical is formed at Y356 via oxidation by the photosensitizer. After the rapid formation of the neutral Y356 radical, the radical is free to propagate in a reversible manner along the pathway composed of Y356, Y731, Y730, and C439, or decay to an off-pathway sink, in both the model and the experiments. Most of the kinetic data were collected for systems with the noncanonical analogs 2,3,6-F3Y or 2,3,5-F3Y at position Y356, but for notational simplicity, this residue will still be denoted as Y356 or Y356* for the reduced or oxidized state, respectively. Moreover, the experiments were performed at pH values for which the Y356 analog is deprotonated to remove the complications associated with proton transfer. For simplicity, our model will not distinguish between electron transfer and PCET, but, rather, will follow the radical as it translocates along the pathway (Fig. 1D).

In the kinetic model depicted in Fig. 1D, we describe the initial radical injection with the rate constant $k_{inj}$ and the subsequent reversible radical transfer steps along the pathway with rate constants $k_1$ to $k_6$. Experiments performed in the presence of Y731F, where transfer into α is blocked by introduction of phenylalanine, revealed a small amount of radical decay to an off-pathway location. This process is incorporated into the kinetic model as $k_{off}$, which could vary for the current systems. Additionally, $k_{PCET}$ is the rate constant that describes the decay of the Y356 radical measured experimentally. Note that this rate constant does not reflect one specific chemical transformation, but, rather, reflects the overall decay of the Y356 radical to the equilibrium populations of all species, where the experimentally measured lifetime $\tau$ is the inverse of $k_{PCET}$.

Prior to giving the details of the modeling procedure, we provide an overview. The injection and off-pathway rate constants $k_{inj}$ and $k_{off}$ are estimated from experimental measurements. The rate constants $k_1$ to $k_6$, which correspond to radical transfer between Y731 and Y730 and between Y730 and C439, are estimated from hybrid quantum mechanical/molecular mechanical (QM/MM) free-energy simulations. The rate constants $k_1$ and $k_5$, which describe the conformational motion of residue Y731, are estimated from classical free-energy simulations. The final two rate constants, $k_2$ and $k_3$, have not been determined directly either experimentally or computationally, but the ranges found must be consistent with the experimentally measured rate constant for Y356 radical decay, $k_{PCET} (1/\tau)$, and the equilibrium constant estimated for this step from experimental data. The values of the known rate constants, which will be discussed further below, are given in Table 1.

Using this model as the base, specific steps along the pathway can be removed by setting their rate constants to zero to model the effect of mutating pathway residues to amino acids that are not redox-active. For example, Y730F corresponds to blocking the pathway at this residue, and C439S corresponds to blocking the pathway at this residue (Fig. 1D). The oxidation of GDP by C439 is not included in the model because $^3$H abstraction by C439 is thought to be slower than the radical translocations probed in the photoRNR experiments (25). The details of the input parameters are provided in SI Appendix, but here we summarize the main elements of the model.
For both the 2,3,6-F₃Y356 and 2,3,5-F₂Y356 photoRNR systems, the Y356 radical lifetimes were measured by using transient absorption spectroscopy for WT-α, as well as with the Y731F and C439S mutations (25, 41) and also with the Y730F mutation for the 2,3,6-F₃Y356 photoRNR system. The value of $k_{\text{off}}$, which is the rate constant for radical injection, was determined by measurement of the excited-state lifetime of the Re photosensitizer in the presence of the Y731F mutation after accounting for other possible decay pathways with information from the Y356F mutation (26). The maximum value of $k_{\text{off}}$, which is the rate constant for the off-pathway radical transfer from Y356, was determined by measurement of the Y356 radical decay in the presence of the Y731F mutation (25, 41). The value of $k_{\text{off}}$ has been hypothesized to be lower when the pathway is fully assembled with an adjacent redox active residue (25). After using the Y731F experimental data to estimate values for $k_{\text{off}}$ and $k_{\text{inj}}$, we investigated the Y356 radical decay for the five available systems: WT-α and C439S for both 2,3,5-F₂Y356 and 2,3,6-F₃Y356 photoRNR, in addition to Y730F for 2,3,6-F₃Y356 photoRNR. The experimental data for all of these photoRNR systems is summarized in Table 1.

The kinetic model can be further determined by using the proposed thermodynamic landscape of the PCET pathway in RNR (42, 43). According to this landscape, the redox potential difference between Y356 and Y731 is ~100 mV, where oxidation of Y731 has been established as thermodynamically uphill through pulsed electron–electron double resonance and EPR spectroscopy (44). This redox potential needs to be shifted to account for the 2,3,6-F₃Y356 and 2,3,5-F₂Y356 mutations. The photochemical experiments on the photoRNR systems were performed at pH 8.2, and, therefore, 2,3,6-F₃Y and 2,3,5-F₂Y are expected to be deprotonated, and the dominant redox couple is (O²⁻/O²⁻), corresponding to electron transfer rather than PCET. The relative redox potentials of tyrosine and these fluorinated derivatives have been measured to millivolt accuracy in a model protein system (43). These redox potentials can be extrapolated to pH 8.2 by using the Nernst equation with the $pK_a$ values measured in a model protein. These extrapolated values are used to shift the redox potential difference between Y731 and either 2,3,6-F₃Y356 or 2,3,5-F₂Y356 to be 60 mV and −10 mV, respectively, corresponding to equilibrium constants for radical transfer from the noncanonical Y356 to Y731 of $K_{\text{eq}}^{1,2} = k_1/k_2 = 10.3$ and 0.68, respectively. Details of these thermodynamic analyses are provided in SI Appendix.

Molecular dynamics simulations (45) based on the cryo-EM structure of the active complex have indicated that Y731 undergoes conformational transitions between a flipped conformation, where it is oriented toward Y356, and a stacked conformation, where it interacts with Y730 (Fig. 1B). Free-energy simulations were performed to compute the relative free energies of these conformations. Due to limitations in the molecular mechanical force field and conformational sampling for this large system, the simulations provided only qualitative insights. For two different choices of reaction coordinates, the relative free energies were determined to be either isoelectric or favoring the flipped conformation by ~4 kcal/mol, corresponding to an equilibrium constant $K_{\text{eq}}^{\text{conf}} = 1$ or 0.001, respectively, at 298 K. The calculated free-energy barriers for this interconversion (45) and previous experimental studies of mutant RNRs (46) show that this motion occurs on a much faster timescale than radical transfer (SI Appendix, Table S3). Thus, we incorporate this conformational motion into our model by assuming that the stacked and flipped conformations remain at equilibrium and that the equilibrium constant is the same for the tyrosine radical and the reduced tyrosine. In this model, Y356 can only engage in radical transfer with Y731 in its flipped conformation, and Y730 can only engage in radical transfer with Y731 in its stacked conformation. Note that these assumptions are not necessary, and the kinetic model could be modified if experimental or computational evidence suggests otherwise. The hydrogen-bonding interactions of the tyrosine radical are different from those of reduced tyrosine, which could impact the conformational motions of this residue. This conformational motion has been shown to be sufficiently rapid to remain at equilibrium in both cases (SI Appendix, Fig. S20), although the equilibrium constants could be different. Distinct equilibrium constants for the tyrosine radical and reduced tyrosine could be incorporated into the kinetic model, if available.

As will be shown by the analysis below, the overall decay of Y356 in the photoRNR systems is relatively insensitive to the values of the rate constants $k_1$, $k_2$, $k_3$, and $k_4$ within physically reasonable ranges. For completeness, however, these parameters can be estimated from QM/MM free-energy simulations. In particular, QM/MM free-energy simulations of the PCET reaction between Y730 and Y731 in the stacked conformation (47) provide estimates of $k_1$ and $k_4$. More recent QM/MM free-energy simulations of the PCET reaction between Y730 and C439 (48) provide estimates of $k_5$ and $k_6$.

The equilibrium between the flipped and stacked conformations does not alter the overall structure of these equations, but simply scales some of the rate constants (Eqs. S3–S8 in SI Appendix).
Our goal is to solve these rate equations to obtain the Y356 radical lifetime for comparison to the experimentally measured lifetimes. To enable a direct comparison, we solve these equations using matrix exponentiation over the same time interval used in the experiment and fit the Y356 radical concentration to an exponential in the same manner as used to fit the experimental data. Specifically, we fit the data between 3 ps and 90 ps or 76.5 ps to a single exponential function for the 2,3,6-F3Y356 or 2,3,5-F3Y356 systems, respectively. We consider a set of rate constants to be consistent with experiment if the lifetime obtained from the fit falls within the reported SD of the experimental value. Moreover, sets of rate constants exhibiting biexponential decay were excluded because they are not consistent with the experimental data.

This kinetic model does not distinguish between electron transfer and PCET, but, rather, describes each step as radical transfer. As mentioned above, radical transfer from the fluorinated Y356 is expected to be electron transfer because the residue would be deprotonated at pH 8.2. The other radical transfer reactions in the subunit are expected to occur by collinear, concerted PCET rather than pure electron-transfer theory. In this context, the radical transfer rates for these steps inherently include proton tunneling and should be interpreted in the context of PCET theory (52, 53), as well as the geometric arrangement of these residues. Thus, the radical transfer rates for these steps inherently include proton tunneling and should be interpreted in the context of PCET theory (52, 53), rather than pure electron-transfer reaction. In this context, the PCET rate constants may be slower than those expected for pure electron transfer due to the factor in the vibronic coupling associated with the overlap between the reactant and product proton vibrational wavefunctions, although the driving force and reorganization energy will also be different.

As discussed above, some of the rate constants cannot be determined directly from experiment or computation, such as $k_1$ and $k_5$, and other rate constants are only determined to be within a certain range due to inherent uncertainties in the experimental or computational methodologies. Moreover, the value of the off-pathway rate constant $k_{off}$ is uncertain for these specific systems. In principle, for each possible value of $k_{off}$, we could determine the combinations of $k_1$ and $k_2$ that satisfy the estimated equilibrium constant for that step and the Y356 radical decay time with all other rate constants fixed. Given the uncertainties in these other rate constants, however, we use a Monte Carlo approach combined with simulated annealing to allow the efficient exploration of the rate constants within the allowed ranges, while satisfying the experimentally known constraints. For the full system, seven rate constants (i.e., $k_{off}$ and $k_1$ to $k_6$) are varied simultaneously. The cost function for the simulated annealing can be constructed from the experimentally measured radical lifetime and the specific constraints. The workflow employed is given in Fig. 2. Full details and validation against the same kinetic model propagated with the Gillespie algorithm rather than matrix exponentiation are given in SI Appendix, Fig. S5.

Analysis of Kinetic Data. These analyses are performed for both the 2,3,6-F3Y356 photoRNR and 2,3,5-F3Y356 photoRNR systems. We start with the 2,3,6-F3Y356 photoRNR system in the presence of the Y730F mutation, which blocks radical transfer between Y731 and Y730, removing $k_3$, $k_4$, $k_5$, and $k_6$ from the model. As discussed above, the maximum of the off-pathway rate constant, $k_{off}$, was determined experimentally for systems containing the Y731F mutation, but this rate constant could be lower when more of the radical transfer pathway is accessible. The values of $k_1$ and $k_2$ are constrained by the range of equilibrium constants implicated by the thermodynamic landscape proposed by analysis of experimental data (42–44). The combined Monte Carlo and simulated annealing procedure can be used to determine values of $k_1$ and $k_2$ that satisfy all of these experimental constraints, as shown in Fig. 3A.

Assuming that the mutations do not significantly alter the structure or provide alternative radical transfer pathways, the fundamental rate constants should be the same for all of the 2,3,6-F3Y356 photoRNR systems studied. Next, we study this system in the presence of the C439S mutation. If we use the values of $k_1$ and $k_2$ estimated from the QM/MM free-energy simulations of PCET between Y730 and Y731, we find that the values of $k_1$ and $k_2$ that satisfy the experimental constraints overlap reasonably well with those obtained for the Y730F mutation. The final system is the WT-α system. If we use the same values of $k_5$ and $k_6$, as well as the values of $k_3$ and $k_4$, estimated from QM/MM free-energy simulations of PCET between Y731 and C439, we also find that the
values of $k_1$ and $k_2$ that satisfy the experimental constraints overlap reasonably well with those obtained for the other mutations.

If we allow the rate constants $k_3$, $k_4$, $k_5$, and $k_6$ to vary over a wide range, we find that the values of $k_1$ and $k_2$ that satisfy the experimental constraints are still in the same range. Thus, the values of $k_1$ and $k_2$ are most important in determining the Y356 radical decay rate in these systems. The following ranges can be assigned based on overlap between the 2,3,6-F$_3$Y photoRNR systems (Table 1). For the 2,3,5-F$_3$Y photoRNR system, however, $K_{eq}^{\alpha}$ is $0.68$ instead of $10.3$, and, therefore, the radical is thermodynamically favored on Y356 instead of Y731. In this case, the values of $k_{\text{eff}}^{\alpha}$ and $k_{\text{eff}}^{\beta}$ are more similar.

The time evolution shown in Fig. 4 was obtained with the same parameters as in Fig. 4 $A$ and $E$, except that $K_{eq}^{\alpha}$, the equilibrium constant for radical transfer from Y731 to Y730, was set to $10$ instead of the value of $8.8 \times 10^2$ obtained from the QM/MM free-energy simulations. This equilibrium constant was decreased by decreasing $k_3$ and slightly increasing $k_4$. The lifetime of Y356 does not change, but now the Y731 radical is observed in appreciable quantities on the 20-µs timescale because the thermodynamic drive from Y731 to Y730 is decreased. In Fig. 4 $B$, the C439 radical still accumulates at a longer, 10-ms time scale, but at a lower concentration. The lower value of $K_{eq}^{\alpha} \times k_{\text{off}}$ leads to more radical transfer from Y730 back to Y731, thereby leading to more radical transfer from Y731 back to Y736, where it can be lost to off-pathway decay. This behavior is further accentuated in the 2,3,5-F$_3$Y system (Fig. 4 $F$), where forward radical transfer from Y356 to Y731 is not thermodynamically favorable, allowing even more radical to be lost to off-pathway decay, resulting in a lower concentration of C439 radical. Thus, the greater value of $K_{eq}^{\alpha}$ can help avoid the nonproductive pathways by enhancing the overall concentration of radical on C439.

The time evolution shown in Fig. 4 $C$ and $G$ was obtained with the same parameters as in Fig. 4 $A$ and $E$, except that the rate constants associated with radical transfer between Y730 and C439 were both significantly increased relative to the values obtained from the QM/MM free-energy simulations. Specifically, $k_3$ was increased by a factor of $100$ from its original value of $1.2 \times 10^3$ s$^{-1}$, and $k_6$ was set to $1.2 \times 10^3$ s$^{-1}$ instead of $5.3 \times 10^2$ s$^{-1}$, resulting in an equilibrium constant $K_{eq}^{\alpha,\beta}$ of $100$ instead of $2.3 \times 10^5$. Now, the radical pools on C439 within $\sim 300$ µs, which is around 100 times faster than with the original
set of rate constants. We found that this time evolution is virtually identical if \( k_6 \) is only increased by a factor of 100, retaining the same equilibrium constant \( K_{eq}^{5,6} \) as determined from the QM/MM free-energy simulations, illustrating that the radical will remain localized on C439 as long as the equilibrium constant is sufficiently large. Thus, the rate constant \( k_5 \) appears to determine the rate of radical transfer to C439 without significantly altering the final concentration within this regime. Increasing this rate constant is a clear strategy for speeding up the overall forward radical transfer in the \( \alpha \) subunit.

The QM/MM free-energy simulations of the PCET reaction between Y730 and Y731 and between C439 and Y730 (47, 48) may have been biased toward forward radical transfer because the subunits of the cryo-EM structure (12) used as the starting point. The QM/MM free-energy simulations of the PCET reaction between Y730 and Y731 and between C439 and Y730 (47, 48) may have been biased toward forward radical transfer because the subunits of the cryo-EM structure (12) used as the starting point.

Fig. 4. Time evolution of radical concentrations on each residue for the 2,3,6-F3Y (A–D) and 2,3,5-F3Y (E–H) systems using four different sets of rate constants for each species. For all 2,3,6-F3Y systems (A–D), \( K_{eq}^{1,2} = 10 \), and for all 2,3,5-F3Y systems (E–H), \( K_{eq}^{1,2} = 0.68 \). For all sets of rate constants, \( K_{eq}^{conf} = 1 \), and the decay of the Y356 radical is in agreement with the experimentally measured value, with the exception of part H, where the Y356 radical does not exhibit single-exponential decay due to rapid back radical transfer. (A and E) Rate constants \( k_5, k_6, k_7, \) and \( k_8 \) were obtained from QM/MM free-energy simulations, and \( k_5 \) and \( k_6 \) were chosen to satisfy the constraints mentioned above. (B and F) Same rate constants as in A and E, except \( k_5 = 5.9 \times 10^5 \text{s}^{-1} \) and \( k_6 = 5.9 \times 10^8 \text{s}^{-1} \), instead of \( k_5 = 5.2 \times 10^5 \text{s}^{-1} \) and \( k_6 = 5.9 \times 10^3 \text{s}^{-1} \). (C and G) Same rate constants as in A and E, except \( k_5 = 1.2 \times 10^5 \text{s}^{-1} \) and \( k_6 = 1.2 \times 10^8 \text{s}^{-1} \), instead of \( k_5 = 1.2 \times 10^5 \text{s}^{-1} \) and \( k_6 = 5.3 \times 10^3 \text{s}^{-1} \). (D and H) Rate constants \( k_5, k_6, k_6, \) and \( k_8 \) were chosen so that \( K_{eq}^{3,4,5} = 1 \) and \( K_{eq}^{5,6} = 0.9 \). In D and H, the red curve is not visually distinguishable from the green curve on the slower timescales. For each case, Left and Right are for shorter and longer timescales, respectively, of the same kinetic simulation. All of the rate constants for this figure are given in SI Appendix, Table S4. Note that the percentages do not add up to 100% because some radical is lost to off-pathway decay. A and C have 1% more C439 radical compared to E and G, respectively, at the final time shown. Analagous plots for \( K_{eq}^{conf} = 0.001 \) and more parameter combinations, as well as the thermodynamic landscapes associated with the parameters used to generate this figure, are available in SI Appendix.
configuration correspond to the preturnover state. Radical equilibration experiments have suggested that the thermodynamic landscape between Y731 and Y730 may be approximately isoergic within experimental error (44). The thermodynamic landscape between C439 and Y730 is unknown, but electrochemical measurements of the cysteine thiyl redox potential in glutathione (54) suggest that radical transfer from Y730 to C439 could be isoergic or slightly thermodynamically uphill (42). Although these latter experiments were not conducted in RNR, we also explored sets of rate constants that are consistent with these implications. For example, we investigated the time evolution of the radical concentration with $K_{eq}^{4} = 1$ and $K_{eq}^{5,6} = 0.9$ for both photoRNR systems (Fig. 4 D and H). For the 2,3,6-F3-Y system (Fig. 4D), the Y731 and Y730 radicals rapidly approach equilibrium with the same concentrations, followed by the rise of the C439 radical. All three radicals have approximately the same concentrations at around 1 ms, given the relatively flat thermodynamic landscape, and then decay to zero as they are lost to off-pathway decay at Y356. Similar behavior is observed for the 2,3,5-F3-Y system (Fig. 4H), except that the thermodynamic landscape is even flatter, with $K_{eq}^{1,2} = 0.68$, and back radical transfer, as well as off-pathway decay, occurs even more quickly. In this case, the Y356 radical decay starts to exhibit biexponential character as compared to the single exponential decay observed experimentally (25), suggesting that such a flat thermodynamic landscape in $\alpha$ is less likely within this model.

Many combinations of rate constants can be explored, and the dependence of the time evolution of the radical flow can be analyzed as a function of each individual rate constant. Additional examples are provided in SI Appendix. These analyses provide fundamental insights into the overall mechanism of radical transfer in RNR and strategies that could be used to alter the rates and final concentrations. To further refine the model and distinguish between the various sets of rate constants, more experimental data that would serve as additional constraints would be helpful. For example, introducing a second spectrally distinct tyrosine analog, in addition to the photochemically oxidized fluorinated Y356, would allow the rate of radical decay to be measured at two sites simultaneously. Although currently this strategy would be very challenging, the field of synthetic biology is rapidly advancing toward making this feasible (55, 56).

**Conclusion**

Herein, we designed a microkinetic model to describe long-range radical translocation in photoRNR systems. In these systems, a radical is injected into RNR through oxidation of Y356 by photoexcitation of a ligated photosensitizer. Our kinetic model includes the injection process, an off-pathway sink, and radical translocation along the pathway composed of Y356, Y731, Y730, and C439, as well as the motion of Y731 during the injection. The injection and off-pathway rate constants are estimated from experimental measurements, the Y731 flipping and stacking rate constants are estimated from classical free-energy simulations, and the rate constants for radical transfer between Y731 and Y730 and between Y730 and C439 are estimated from QM/MM free-energy simulations. The rate constants that describe forward and reverse radical transfer between interfacial residues Y356 and Y731 are obtained by constraining the Y356 radical decay time to the values obtained from experimental photochemical measurements and the equilibrium constant to be within the experimentally determined range. This procedure provides estimates of the ranges for these two critical rate constants, which were found to be consistent across several mutant photoRNRs for each of the two fluorinated Y356 residues studied. Specifically, the effective rate constants were determined to be in the range of $k_{eff}^{1} = 5.5 \times 10^{-3}$ to $7 \times 10^{-2}$ s$^{-1}$ and $k_{eff}^{2} = 5.0 \times 10^{-3}$ to $6.0 \times 10^{-2}$ s$^{-1}$ for 2,3,6-F3-Y photoRNR and $k_{eff}^{1} = 7.0 \times 10^{-3}$ to $6.8 \times 10^{-2}$ s$^{-1}$ and $k_{eff}^{2} = 1.1 \times 10^{-2}$ to $1.0 \times 10^{-2}$ s$^{-1}$ for 2,3,5-F3-Y photoRNR. Specific values can be obtained for a desired value of $k_{eff}$ in Fig. 3. These rate constants are not experimentally accessible due to the complexity of the process.

Most importantly, this kinetic model reveals the time evolution of radical transport along the tyrosine and cysteine residues following radical injection. In this manner, the time dependence of the concentration corresponding to the radical located on each residue along the pathway can be visualized. Given the uncertainties in the experimental and simulation methodologies used to determine the input quantities, we conducted a comprehensive analysis of the sensitivity of the radical translocation process on the individual rate constants. This analysis identified the rate constants that may be tuned to alter the radical concentration on a specified residue on a specified timescale. Specifically, the rate constants can be tuned to increase the final concentration of radical on C439 or to speed up the time required for the radical to equilibrate on C439.

This kinetic model is sufficiently general that it can also be used to investigate a wide range of other design principles for enhancing the catalytic efficiency or specificity of RNR. Moreover, this model may be extended to include other portions of the radical transfer pathway and additional protein conformational motions. It could also be used to predict the changes in radical lifetimes and populations upon introduction of a fluorinated tyrosine or other mutation, either along the pathway or near the pathway. These predictions could be tested experimentally, and the feedback between the kinetic modeling and the experiments would assist in refining the model and increasing the overall understanding of the radical transfer process in RNR. Moreover, this general kinetic modeling strategy, utilizing input and constraints from both experimental measurements and simulations, is broadly applicable to other biological processes.

**Data Availability.** Additional kinetic analysis and discussion of input parameters is provided in the SI Appendix. Code and raw data have been deposited in the Open Science Framework (DOI: 10.17605/OSF.IO/XDHET) (57).

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