Ribonucleotide reductases (RNRs) catalyze the conversion of four nucleoside di- or triphosphates (ND(T)Ps) to deoxyribose-nucleoside di- or triphosphates (dND(T)Ps) in all organisms (Figure 1).1–3 RNRs are highly regulated enzymes playing an important role in controlling the ratio and relative amounts of dNTPs essential for the fidelity of DNA replication and repair. Imbalance in dNTP pools results in genomic instability and leads to disease states.4–6 RNRs’ essential role has made them targets for cancer and, more recently, antibiotic therapeutics.6–12

The E. coli class Ia RNR, a prototype model system for human RNR,6 is composed of two subunits, α3 and β6, both required for activity. Based on their α3 and β6 structures, Uhlin and Eklund proposed a symmetrical αβ docking model (Figure 2A) for active RNR, which has played a central role in the experimental design.13 The model for substrate activation and chemistry requires that the diferric tyrosyl radical (Y122*) of 3,5-F2Y731 may act as a bridge between Y356 and Y731, as shown in Figure 1C. Y356, a tyrosyl residue located in the α3 subunit, has been shown to play a crucial role in thiol radical formation.15

1. INTRODUCTION

Ribonucleotide reductases (RNRs) catalyze the conversion of four nucleoside di-or triphosphates (ND(T)Ps) to deoxyribonucleoside di- or triphosphates (dND(T)Ps) in all organisms (Figure 1).1–3 RNRs are highly regulated enzymes playing an important role in controlling the ratio and relative amounts of dNTPs essential for the fidelity of DNA replication and repair. Imbalance in dNTP pools results in genomic instability and leads to disease states.4–6 RNRs’ essential role has made them targets for cancer and, more recently, antibiotic therapeutics.6–12

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Despite much insight into nature’s design for radical initiation in RNRs, elucidating the molecular basis for the RT across the α/β subunit interface has been hampered by the lack of structural information about the C-terminal tail of all βs (residues 341–375 in E. coli RNR), essential for α/β subunit interaction.35−37 The location of Y356 in the RT pathway within this tail was thus unknown. Recently, a near-atomic resolution cryo-EM structure of a trapped αβ2 E. coli complex was obtained (Figure 2B).38 It was generated from the incubation of a double mutant of β2,E52Q/F3Y122−β2, with wt-α2, GDP (substrate) and allosteric effector (TTP) with freeze-quenching at 50 s. The 2,3,5-F3Y122 substitution allowed the generation of one dGDP product and accumulation of one pathway radical at Y356•. The E52Q mutation was important for successfully trapping the αβ2 complex. The E52 residue resides transient absorption spectroscopic methods using photo-β2 RNRs.30−34

Figure 1. Reduction of NDPs to dNDPs catalyzed by Escherichia coli class Ia RNR. The reduction is initiated by a thyl radical (C439•), and the reducing equivalents are provided by the oxidation of C225 and C462 to a disulfide. Multiple turnovers require a redoxin reducing system such as thoredoxin (TR), thoredoxin reductase (TRR), and nicotinamide adenine dinucleotide phosphate (NADPH).

Figure 2. Docking model13 (A) and cryo-EM structure38 (B) of the αβ2 complex of E. coli class Ia RNR and the proposed RT pathway, (C) and (D), respectively. (A) The docking model based on the shape complementarity of subunits α213 and β214 (B) Cryo-EM structure of an αβ2 complex of RNR generated when E52Q/F3Y122−β2, wt-α2, GDP (substrate) and TTP (effector) were quenched at 50 s (pdb code: 6W4X).38 Asymmetry of the complex is indicated by α′β′ (disordered pair) and αβ (ordered pair). (C) The proposed forward RT pathway based on many experiments.20−27,30−33 W48 is shown in parentheses as there currently is no direct evidence for its involvement. The red and blue double arrows describe electron and proton transfers, respectively. Evidence for the bold water molecules has been reported recently.27,28 (D) An intact RT pathway within αβ including Y356 and its position relative to Y731 is visible for the first time in the cryo-EM structure.38 Distances between RT residues are indicated; the 19F atoms of 2,3,5-F3Y122 present in the cryo-EM structure have been omitted. Interfacial residue Q52 (E52 in wt-RNR) is included as it was important for stabilizing the αβ2 complex in the cryo-EM experiment.
at the α/β-interface and is essential for activity, enabling proton release during Y356 oxidation in the RT.\textsuperscript{13,59}

The cryo-EM structure (Figure 2B) revealed an asymmetric αβ complex, consistent with earlier results\textsuperscript{37,40} It also revealed the residues in the C-terminal tail of β (341–375) in an ordered αβ pair, the intact RT pathway including the location of Y356 and its location relative to Y731 (α) (Figure 2D) for the first time. The entire C-terminal tail in α/β, where chemistry has occurred and Y356 is supposedly trapped, remains disordered.

The importance of Y356 during RT has been established by many different methods that often led to the detection of the Y356* intermediate. Recent studies to identify the proton acceptor during its oxidation in forward RT revealed that the most reasonable candidates, E52β and E350 (β), both conserved and essential\textsuperscript{36,39,41}, are unlikely to be the ultimate acceptors.\textsuperscript{33,34,42} These residues are located at ∼7 Å (E52) and ∼14 Å (E350) distances from the phenol-oxygen atom of Y356 in the ordered αβ pair of the cryo-EM structure,\textsuperscript{38} too far for direct proton or H atom transfer with Y356. A variety of H and 17O high-frequency electron-nuclear double resonance (ENDOR) experiments on Y356\textsuperscript{14} and\textsuperscript{27,28} kinetic studies using RNRs with F3Y122\textsuperscript{33} and a photo-oxidant appended to the C555 mutant of β, and pH studies of Y356 formation using F2Y356 all support the interaction of Y356* with water (Figure 2C).

Efforts to understand the residues involved in managing the proton to support the PCET between Y356 and Y731 across the α/β interface have been less successful. The cryo-EM structure shows an O–O distance between Y356 and Y731 of ∼8 Å in ordered αβ, with Y356 in its unusual stacked conformation with Y731 as in previous X-ray structures of αβ\textsuperscript{2,13} While a number of pulsed electron double resonance (PELDOR) experiments\textsuperscript{27,28} revealed sharp distance distributions consistent with little Y356* flexibility, several different experiments reported the mobility of Y731. In a crystal structure of NH2Y730αβ, Y731 was found in a conformation where it is flipped away from the stacked conformation with NH2Y730\textsuperscript{44} PELDOR studies on a double mutant R411A-NH2Y731αβ under turnover conditions revealed a conformational change of 3 Å in trapped NH2Y731\textsuperscript{*, consistent with a flipping toward the α/β interface.\textsuperscript{43} Subsequent studies using photo-β to the same αβ mutations revealed dynamic/rapid conformational changes of Y731.\textsuperscript{40} Another EPR study by Yokoyama et al. suggested the flipping of F3Y122\textsuperscript{2,23} which was trapped as a minority radical species in NOY122β/F3Y122αβ. Molecular dynamics (MD) simulations using the cryo-EM structure and the α/β interface in water also support the flexibility of Y731\textsuperscript{45} with movement away from the stacked conformation with Y730. The studies together support a model for PCET between Y356* and Y731 across the α/β interface that could involve a movement of Y731 toward the interface (Figure 2C), with consequences for their PCET chemistry. However, structural or spectroscopic evidence for interaction between Y356* and Y731 has never been observed.

In this article, we use 19F–Y analogues introduced site-specifically into E. coli RNR, F2Y122β (or the double mutant F352Q/F2Y122β), incubated with 3,5-F2Y356αβ GDP, and TTP to generate and trap Y356* F2Y731 was chosen for its symmetric 19F substitution pattern and minimally perturbed reduction potential relative to \textsuperscript{19F}.\textsuperscript{46,47} The Y356 location and identity are established using 34 GHz PELDOR and 263 GHz EPR spectroscopies, respectively. \textsuperscript{19F} ENDOR spectroscopy\textsuperscript{48,49} at 94 GHz is used in an effort to determine the distances across the subunit interface between the trapped Y356* (β) and the \textsuperscript{19F} nuclei of F2Y731(α). The ENDOR spectra give unambiguous evidence for two conformations of F2Y731*. One conformation is consistent with the structure observed by cryo-EM (ordered α/β pair). The second conformation indicates a flipping of F2Y731 toward Y356*. The results have important implications for the PCET mechanism across the α/β interface.

2. MATERIALS AND METHODS

2.1. Preparation of RNR Mutants and Activity Assays. The RNR mutants F1Y122β, E52Q/F1Y122β, F352Y356αβ, and \textsuperscript{3,5}O–wt-αβ were expressed and purified, as previously described.\textsuperscript{50,51} Activities of (E52Q)F1Y122β/F1Y731αβ and wt-β/F3Y122O–Y731 were determined using the spectrophotometric assay (Supporting Information (SI) 1, Table S1).\textsuperscript{51}

2.2. EPR Sample Preparation. The Y356* intermediate was trapped by incubating a solution of F2Y356αβ, GDP, and TTP in assay buffer (50 mM HEPES, 15 mM MgSO\textsubscript{4}, 1 mM EDTA, pH 7.6) with F1Y122β or E52Q/F1Y122β in assay buffer. Glyceral concentrations were optimized (Figure S1) and typically added to ∼20% of the final volume to prolong phase memory times T\textsubscript{M} for PELDOR and ENDOR measurements. The final concentrations were ∼80 μM αβ, ∼1 mM GDP, and ∼200 μM TTP. The reaction mixture was transferred to either 34 GHz EPR tubes (Q-band) (12 μl, 1.5 mm inner diameter (ID) Suprasil tube, Willmar) or 94 GHz (W-band) tubes (4.4 μl, 0.7 mm ID fused silica quartz tubes) and quenched by freezing in liquid nitrogen at reaction times (T\textsubscript{Q}) of 40–80 s (Q-band) or 35–55 s (W-band). A second set of samples were prepared with T\textsubscript{Q} > 100 s. Two hundred and sixty-three GHz EPR samples were prepared in Suprasil capillaries (ID 0.2 mm, Vitrocom) without glyceral and quenched at T\textsubscript{Q} = 15–20 s. All samples are summarized in SI 2, Table S2.

2.3. 263 GHz EPR Spectroscopy. High-frequency (HF) 263 GHz echo-detected EPR spectra were recorded with a commercial spectrometer, as previously reported.\textsuperscript{27} Details on the spectral acquisition are given in SI 3.

2.4. 34 GHz PELDOR Spectroscopy. Four-pulse PELDOR experiments\textsuperscript{53,54} were performed at 34 GHz (Q-band) on a commercial Bruker ELEXSY E580 EPR spectrometer, as previously reported.\textsuperscript{27} An optimized temperature of 50 K was selected, where high sensitivity is achieved and unreacted F3Y122O– does not contribute to the spin echo under conditions used for data collection (SI 4.1–4.3). MW pulses were amplified by a pulsed 170 W TWT amplifier (Model 187K, Applied Systems Engineering) with typical pulse lengths of 14–16 ns for the pump π-pulse at the center of the overcoupled resonator. The observer frequency was set to −105 MHz from the dip center, leading to observer π-pulse lengths of 24–28 ns. The τ\textsubscript{Q} value was 250 ns, and τ\textsubscript{E} values were optimized based on T\textsubscript{M} measurements (SI 4.2). Shot repetition times were 4–6 ms. Time traces were recorded at three different observer positions (Figure S5) and their intensities were summed, reflecting their respective EPR signal strengths at that excitation position. Traces were analyzed with DeerAnalysis 2019,\textsuperscript{55} using Tikhonov regularization (L-curve criterion for α parameter) and checked for consistency using neural network analysis.\textsuperscript{6,57}

2.5. 94 GHz ENDOR Spectroscopy. Pulsed EPR and ENDOR experiments at 94 GHz (W-band) were performed on a commercial Bruker ELEXSY E680 EPR spectrometer, as previously described.\textsuperscript{27} Using a 2 W MW amplifier, typical π/2 pulse lengths of 10–12 ns were achieved. EPR (echo-detected) spectra and signal contributions are illustrated in SI 5.1. Shot repetition times were optimized to 2–4 ms based on T\textsubscript{M} measurements (SI 5.2).

\textsuperscript{19F} Mims ENDOR spectra of the Y356* were recorded using radio frequency (RF) pulses amplified by a 250 W RF amplifier (250A250A Amplifier Research). RF pulse lengths of 22 μs were used for \textsuperscript{19F} nuclei with ∼1.6 MHz couplings or 44 μs for couplings ≤250 kHz. RF pulse lengths were optimized using Rabi nutation experiments. Stochastic RF acquisition\textsuperscript{58–60} with 20 shots per point was used. To observe \textsuperscript{19F} couplings of different sizes, the adjustment of the
interpulse delay $\tau$ in the Mims sequence was crucial. For couplings on the order of 1.6 MHz, two measurements with $\tau$ values of 236 and 266 ns were performed and summed subsequently (normalized to the number of scans) to attenuate the proton background. For smaller couplings, $\leq 250$ kHz, $\tau$ was optimized to 620–622 ns (SI 5.3).

ENDOR spectra were recorded at three different observer positions (Figure S8) and summed up with intensities reflecting their respective EPR signal strengths at that excitation position.

Data were collected at two temperatures. At 50 K, EPR sensitivity was higher than that at 80 K, where usually the signal of unreacted F3Y122 disappears due to faster relaxation. As a downside, at 50 K, the unreacted F3Y122 contributed to the echo intensity of the Mims sequence at short interpulse delays $\tau$. The contribution of F3Y122 led to $^{19}$F ENDOR background signals, which had to be removed during data processing (SI 5.4). As a control for the background correction procedure, we repeated representative $^{19}$F ENDOR measurements at 80 K (SI 5.5–5.6) where no background of F3Y122 was present. The results obtained at 50 and 80 K are fully consistent. In addition to the $^{19}$F background, broad, overlapping $^1$H resonances associated with the 3,5-H atoms of Y356 were identified by their changes associated with $\tau$ value changes and they were subtracted from the $^{19}$F spectra, as illustrated in SI 5.4.

$^{17}$O ENDOR control experiments were performed using similar parameters described in our recent $^{17}$O ENDOR study$^{28}$ and are reported in SI 6.  

2.6. Simulations of ENDOR Data. Mims ENDOR simulations of the Y356$^{\alpha}$ were performed using EasySpin's saffron routine.$^{61}$ The g tensor was $g_x = 2.0062$, $g_y = 2.0044$, and $g_z = 2.0022$. In the molecular frame, $g_z$ is aligned along the C=O$^*$ bond of Y356$^{\alpha}$, while $g_x$ is perpendicular to this direction and in the plane of the aromatic ring. The strongly coupled $\beta$-proton of Y356$^{\alpha}$ was included using previously reported hyperfine coupling (HFC) parameters.$^{27}$ For simulating the $^{19}$F ENDOR spectra with $\tau = 620–622$ ns, the C3 and C5 protons of Y356$^{\alpha}$ were included. The $^{19}$F ENDOR line width parameter was simulated as 25 kHz for couplings below 0.5 MHz.$^{60}$ For larger couplings, a line width of 250 kHz was used. Chemical shift anisotropies were not resolved in the 94 GHz $^{19}$F ENDOR spectra.$^{62}$

2.7. Structural Models for ENDOR Analysis. Due to the large parameter space associated with the two Fs of F3Y731 and, as well as become clear, their multiple side-chain conformations, a fitting routine that generates the most likely set of HFC parameters by minimizing residuals (rmsd) is not possible. We therefore used an approach similar to that described previously to analyze the PCET steps with $\alpha_\beta$ using NH2Y356 and the X-ray structure of $\alpha_\beta$ to position Y370 and C439.25 In the present case, the small models were constructed starting from pdb 6W4X, the current cryo-EM structure (resolution 3.3–5.5 Å).$^{38}$ Y356 from $\beta$ and Y371 from $\alpha$ were extracted from the ordered position Y730 and C439.$^{25}$ In the present case, the small models were constructed starting from pdb 6W4X, the current cryo-EM structure (resolution 3.3–5.5 Å).$^{38}$ Y356 from $\beta$ and Y371 from $\alpha$ were extracted from the ordered position Y730 and C439.$^{25}$ In the present case, the small models were constructed starting from pdb 6W4X, the current cryo-EM structure (resolution 3.3–5.5 Å).$^{38}$ Y356 from $\beta$ and Y371 from $\alpha$ were extracted from the ordered position Y730 and C439.$^{25}$

3. RESULTS

3.1. Characterization of RNR Constructs Using Activity Measurements, High-Field EPR, and PELDOR. The first part of the investigation required examination of the new RNR constructs that contain the $^{19}$F labels in F2Y731. Steady-state activities are reported in Table S1. Spectrophotometric assays revealed a specific activity of 560 nmol/(mg-min) (ca. 7% of wt) for F3Y122$^{\alpha}$/F2Y731$^{\alpha_\beta}$, defined with respect to the mass of $\beta_\alpha$ in the assay. In contrast, an activity of only 6 nmol/(mg-min), that is, the lower limit of detection, was measured for E52Q/F2Y122$^{\alpha}$/F2Y731$^{\alpha_\beta}$. The latter finding was expected, as the E52Q mutation disrupts steady-state activity.$^{39}$ Nevertheless, both constructs are capable of one turnover and allowed trapping of the intermediate Y356$^{\bullet}$ for EPR samples during back-radical transfer.$^{67}$ Moreover, glycerol is required in the sample preparation to prolong spin relaxation in the EPR experiments. Thus, the glycerol content (v%) was also optimized based on its effect on RNR activity (SI 1) and a value of 20 v% was selected for almost all samples (SI 2, Table S2). We characterized the structure of the trapped radical in F2Y122$^{\alpha}$/F2Y731$^{\alpha_\beta}$ and E52Q/F2Y122$^{\alpha}$/F2Y731$^{\alpha_\beta}$ by 263 GHz EPR (SI 3). In all quenched reaction mixtures, two radical species were observed (Figure S2). One contribution arose from the unreacted F2Y122$^{\bullet}$ and was readily identified by its large $g_z$ value (2.0082) and its characteristic $^{19}$F HFC structure. After subtracting a reference spectrum of F3Y122$^{\bullet}$, the spectrum of the intermediate became visible (Figure S3). This radical was identified as Y356$^{\bullet}$ due to the characteristic low $g_z$ value of 2.0062 (reference spectrum of Y356$^{\bullet}$ is shown in Figures S2 and S3), as reported with F3Y122$^{\alpha}$/Y356$^{\alpha_\beta}$ (SI 3). The analysis of the HF-EPR spectra also revealed no other radical species.

PELDOR spectroscopy (34 GHz) was then used to measure the diagonal distance between Y356$^{\bullet}$ and one of the $\alpha_\beta$ pairs F2Y122$^{\bullet}$ in the second one (Figure 4). The orientation-averaged time traces exhibit clear oscillations. Indistinguishable results were obtained for various sample preparation conditions (SI 4). For comparison, a time trace of F2Y122$^{\alpha}$/F2Y731$^{\alpha_\beta}$ was also measured (Figure 4, green). Distance distributions with a single peak centered at 3.03 ± 0.02 nm (Figure 4) and a width (full width at half-maximum (FWHM); Table S4) of 0.09–0.14 nm were obtained for all samples. The observed distance is typical for F2Y122$^{\bullet}$—Y356$^{\alpha_\beta}$ pairs.$^{23,27}$ From PELDOR and HF-EPR, we
PELDOR distance should be between Y356 and the cryo-EM structure (Figure 2B), then the observed

$$\sim$$

Additionally, sharp features are observed in a

$$\nu$$

around 0.8 MHz in both samples. However, in the cryo-EM structure, the C-terminal $$\beta$$ tail is disordered at the interface, indicating that the trapped state might be different under the conditions of the EPR experiments. Because of the disorder, the distance between F3Y122 $$\alpha$$ and Y356 $$\beta$$ cannot be measured in the cryo-EM structure. If we consider the opposite diagonal distance, i.e., between the centroids of Tyr-O, C1, C3, and C5 atoms of F3Y122 $$\beta$$ and Y356 $$\alpha$$, then the PELDOR distance of 3.0 nm is in agreement with this structure. We note that many such distances have been measured with other constructs. All give a sharp 3 nm distance feature, suggesting that the Y356 conformation is constrained. Our model for half-site RNR reactivity requires that the complex interconverts to allow for alternating PCET in $$\alpha$$ and $$\beta$$. When the Y356 $$\alpha$$ is trapped, the interconversion is slow. The kinetics of this structural interconversion and the mechanism of switching remain to be established but are likely to be critical for comparing results from different experimental setups.

### 3.2. Distance Measurements across the RNR $$\alpha$$/$$\beta$$ Interface Using 94 GHz 19F ENDOR

#### 3.2.1. 19F ENDOR Detects Y356*–F2Y731 Distances

19F ENDOR spectra of Y356* in F3Y122/F2Y731–$$\alpha$$ (black) and E52Q/F3Y122/F2Y731–$$\alpha$$ (red) were obtained after summing three background-corrected, orientation-selective spectra in the range of ±4 MHz around the 19F Larmor frequency $$\nu_0$$ (19F) (Figure 5A). When using short $$\tau$$ values (236 and 266 ns), prominent resonances are observed at ±0.8 MHz in both samples. These resonances are attributed to one 19F nucleus, $$F_w$$, with a peak separation of ~1.6 ± 0.1 MHz (purple, dashed lines). Additionally, sharp features are observed in a ±250 kHz region around $$\nu_0$$ (19F). These resonances were investigated using a larger $$\tau$$ value of 620 ns, which enhances the sensitivity for smaller couplings (Figure 5B). For both samples, the spectra in Figure 5B can be interpreted as a superposition of two Pake patterns contributed by two 19F nuclei, designated as $$F_b$$ and $$F_c$$. Pake patterns result from purely dipolar coupling and allow assignment of the corresponding dipolar HFC $$T$$ by reading off the splitting between the sharp, central peaks: $$T_b = 250 \pm 15$$ kHz (cyan, dashed lines) and $$T_c = 150 \pm 15$$ kHz (green, dashed lines). These peaks are contributed by molecules in which the 19F-radical interspin vector is perpendicular to the external magnetic field $$B_0$$. Using the point-dipole approximation (eq 1)

$$T = T_1 = \frac{74.52}{R^3}$$

we can estimate interspin distances of 6.7 ± 0.2 Å and 7.9 ± 0.3 Å, with the centroid of the O1, C1, C3, and C5 atoms of Y356* as a point of reference. Aside from the central peaks, Pake patterns are also characterized by shoulders appearing at twice the coupling strength (2$$T = T_0$$). These features are contributed by molecules with interspin vectors parallel to $$B_0$$. The dipolar approximation does not apply for the stronger coupling $$T_0$$ due to the shorter distance, <5 Å.

The observation of three distinct 19F resonances in Figure 5A,B requires at least two conformations of F3Y731. Since each conformation contributes two 19F–Y356* spin pairs, a fourth set of resonances ($$F_d$$) is expected but not clearly resolved in the spectra obtained by summing up three orientation-selective measurements. An indication for coupling to a fourth nucleus $$F_d$$ was provided by the orientation-selective measurements with $$B_0$$ aligned along $$g_c$$ (Figure 5C). Here, strong selectivity for the parallel components of $$F_b$$ and $$F_c$$ was observed. In addition, shoulders on the inside of the two most prominent features are observed, which suggest the parallel coupling of the fourth atom $$F_d$$. Further analysis of the orientation-selective spectra is discussed below and will confirm this assignment.

Interestingly, the size of the observed HFCs (peak positions) is conserved in both F3Y122/F2Y731–$$\alpha$$ and E52Q/F3Y122/F2Y731–$$\alpha$$ mutants, but the spectrum of E52Q/F3Y122/F2Y731–$$\alpha$$ in Figure 5A appears broader, suggesting more heterogeneity in this mutant.
green color in Figure 3 (Table S6). This resulted in model S2, illustrated in Figure 6. We note that in model S2, as well as in all other models, a water molecule was introduced in the vicinity of Y356* (Section 2.7), the presence of which was reported earlier.27,28 The H-bonding water molecule affects Y356*'s spin density distribution and, consequently, also the effective 19F-radical HFCs. As detailed in SI 7, the resulting geometrical changes are minor and amount to ca. 0.1–0.2 Å.

In S2, the 19F–Y356* distances are 9.8 and 8.4 Å, the latter consistent with the estimate for Rb based on the dipolar approximation (eq 1). DFT analysis of S2 predicts coupling constants of 85 and 153 kHz, reproducing the coupling of Fc in Figure 5B within the estimated uncertainty. The 85 kHz coupling could be attributed to Fc. When the triad shown in S2 is incorporated back into the cryo-EM structure, the position of Y356* was found to fulfill the PELDOR diagonal distance of 3.0 nm (Figure 4 and Table S7).

Nevertheless, it is clear that neither model S2 nor reorienting the ring plane of Fc (model S3, Figure S16) is able to reproduce the observed strong HFCs of Fc.

Therefore, we examined the possibility that a second conformation between the interfacial Ys might result in a second pair of stronger 19F HFCs. This proposal is reasonable based on previous evidence from different types of experiments that Y731 can flip.23,26,30,44,45 A small model based on the flipped Y-dyad taken from the X-ray structure of NH2Y730-44 (without β) could not be placed into the cryo-EM structure using pair fitting (in PyMOL) of the ring atoms to superimpose the Y730 side chains since clashes resulted (SI 8, Figure S17). This is in principle expected because this structure is missing the β subunit, which provides structural constraints. We thus focused on αβ and returned to model S2, adjusted the dihedral angles around Cα–Cβ and N–Cα of Y731 (Table S6), until the DFT-predicted HFC couplings reached the range of the experimental values for Fc and Fb. Representative structures that fulfilled the 19F HFCs are shown as models S4 and S5 (Figure 7), in which the fluorophenol groups are flipped by about 50–70° toward the subunit interface.

In S4 (Figure 7A,C), the 19F nuclei reside at distances of 4.1 and 6.8 Å from the centroid of Y356*. For the proximal 19F atom (Fb), DFT predicts a dipolar coupling constant Tc of ~1.0 MHz and a negative, isotropic coupling constant 2<sub>iso,a</sub> of ~0.8 MHz. This combination leads to a splitting of ~1.8 MHz for S4, similar to the ~1.6 MHz observed experimentally for Fc (Figure 5A). The larger of the two 19F-radical distances in S4 agrees well with the estimate for Rb, yielding a coupling

3.2.2. Examination of Structural Models of the Triad Y<sub>356</sub>–F<sub>2</sub>Y<sub>731</sub>–Y<sub>356</sub>. To rationalize the 19F ENDOR spectra, structural models of the tyrosine triad were built (Section 2.7 and Figure 3) and the DFT-predicted 19F HFCs were compared with the experimental values in Figure 5. The starting point for modeling is the cryo-EM structure.30 Model S1 (Figure 3, black) is identical to this structure, with two 19F nuclei replacing the 3,5-H atoms in Y731. This structure results in HFCs of 65 kHz and 114 kHz (see also SI 8, Table S8), the latter approaching but not quite matching the 150 kHz indicated for Fc in Figure 5B given DFT uncertainties up to 20%. The 65 kHz coupling could potentially be attributed to the fourth 19F nucleus, Fb.

To increase the coupling strength in S1, either the position of F<sub>2</sub>Y<sub>731</sub> or of Y<sub>356</sub>* had to be readjusted for the spin centers to come closer. An increase of T<sub>c</sub> from 114 to ~150 kHz for Fc would require reducing the internuclear distance by roughly 1 Å based on eq 1. To maintain the stacked arrangement of F<sub>2</sub>Y<sub>731</sub> and Y<sub>730</sub> observed in almost all available structures, we adjusted the position of O–Y<sub>356</sub>* by ca. 1 Å, which is still well within the resolution of the cryo-EM structure, as indicated in Figure 5. The larger of the two 19F-nuclear distances in S1 is ~1.0 MHz and a negative, isotropic coupling constant 2<sub>iso</sub> of ~0.8 MHz. This combination leads to a splitting of ~1.8 MHz for S4, similar to the ~1.6 MHz observed experimentally for Fc (Figure 5A). The larger of the two 19F-radical distances in S4 agrees well with the estimate for Rb, yielding a coupling
constant $T_b$ of 254 kHz, in agreement with the resonances of $F_b$ (Figure 5B).

In a second model with a flipped Y731 (SS, Figure 7B,D), a distinct orientation of Y731 and Y356 was considered to account for orientation selection (see also next section). In SS, the $^{19}$F−Y356$^*$ distances are 4.6 and 7.3 Å. The interspin vector from the distal $F_b$ to the centroid of $Y_{356}^*$ is nearly parallel to the direction of $g_x$ (Figure 7D) and distinct from S4 (Figure 7C). It has a DFT-derived HFC of $T_b = 246$ kHz. For the proximal $^{19}$F nucleus $F_a$, a dipolar coupling constant of $T_a \approx 0.8$ MHz with a negative isotropic coupling constant $\alpha_{aa}$ of $-1.0$ MHz is predicted and leads to an expected peak separation of $\Delta v = 1.8$ MHz as in S4.

A comparison of DFT-predicted HFCs from all models, S1–SS, and the experimental values is shown in Figure 8. More details on geometrical parameters of the five models are summarized in Table S7. We note that the combination of S2 with either S4 or S5 could satisfy the experimentally observed peak separations in Figure 5.

Finally, both S4 and S5, when integrated back into the framework of the cryo-EM structure, give centroid–centroid distances between $F_2Y_{731}$ and $F_3Y_{122}$ of $35.0$ and $35.5$ Å, respectively, both very similar to the constraints measured in our previous PELDOR experiments. 

3.2.3. Spectral Simulations Including a Superposition of Stacked and Flipped Y731 Conformations. The DFT analysis indicated that it is possible to find mutual conformations of $F_2Y_{731}$ and $Y_{356}^*$ which individually satisfy some observed $^{19}$F−$Y_{356}^*$ distances. To examine whether a superposition of these conformations can reproduce the ENDOR spectra, we also considered the orientation-selected ENDOR spectra, which pose additional constraints with respect to the sum spectra of Figure 5.

Representative orientation-selected spectra, corresponding to the black sum spectra of Figure 5, are displayed in Figure 9. In the small coupling region (Figure 9B), we observe that $T_{bb}(F_b)$ appears enhanced at $g_x$, suggesting an orientation of the $F_b$ dipolar tensor parallel to $g_x$. Therefore, a structure similar to S5 likely describes the data better than S4, as illustrated in Figure 7C,D, where the orientation of the dipolar vector with respect to $g_x$ is displayed.

Using these orientational constraints, global simulations of the orientation-selective ENDOR spectra based on models S2 and S5 were carried out with the DFT-predicted parameters listed in Table 1 and the ratio (i.e., the relative contribution of S2 and S5) varied until a minimum of residual could be found (SI 9). rmsd from these simulations for all samples amount to ca. 0.1 or 10% at the optimized ratios (Figure S18). We observed that the simulation of the large coupling $F_a$ (Figure 9A) is not very sensitive to the weighting of $F_a−F_d$, and the simulations of the small coupling region, as can be seen in Figure 9B by the decomposition of the simulation into the individual contributions. We note that the obtained weighting of the flipped conformation slightly varies between samples from 18 to 33% within an error of 5% for each sample (Table 2). Therefore, we estimate that the flipped conformation represents on average 25 ± 10% of the molecular ensemble.
Figure 9. 94 GHz $^{19}$F Mims ENDOR spectra on F$_3$Y$_{122}$-$\beta_1$/F$_3$Y$_{731}$-$\alpha_2$ (80 $\mu$M, T$_Q$ = 50 s) at T = 50 K. (A) Measurement with short $\tau$ values ($\sim$250 ns). (B) Measurement with larger $\tau$ values ($\sim$620 ns). Simulations including four different $^{19}$F atoms (F$_1$–F$_4$) are shown as blue lines and are based on S2 and S5 (Tables 1 and 2). Contributions of individual $^{19}$F atoms are shown as shaded areas: purple (F$_a$), cyan (F$_b$), green (F$_c$), and brown (F$_d$).

Table 1. Parameters Used for the ENDOR Simulations

| atom (model) | F–Y$_{356}$ * [Å] | $A_{iso}$ | $A_{a}$ | $A_{b}$ | $A_{c}$ | $A_{iso}$ [kHz] |
|--------------|------------------|----------|---------|---------|---------|-----------------|
| F$_1$ (S5)   | 4.6              | 580, −1668, −1952 | 1013     |
| F$_2$ (S5)   | 7.3              | −246, −246, 492   | 0        |
| F$_3$ (S2)   | 8.4              | −159, −159, 318   | 0        |
| F$_4$ (S2)   | 10.0             | −83, −83, 166    | 0        |

“Distances defined with respect to the centroid of Y$_{356}$*, as shown in Figures 6 and 7B. *Coupling constants $A_i$ consider the anisotropic and the isotropic coupling constants ($T_i$ and $a_{iso}$, respectively): $A_i = T_i + a_{iso}$. Euler angles for relating the $A$ to $g$ tensors are reported in Table S8. An error of ±1 kHz was estimated for couplings <500 kHz, while an error of ±125 kHz is estimated for the 1.6 MHz coupling (ca. 50% of the ENDOR line width parameter in both cases).

Table 2. Ratios of the Stacked Model S2 and the Flipped Model S5 from ENDOR Simulations

| RNR mutant | $T_Q$ [s] | contribution of flipped (S5) * |
|------------|-----------|--------------------------------|
| F$_2$Y$_{122}$-$\beta_1$/F$_2$Y$_{731}$-$\alpha_2$ | 50        | 33%                            |
| F$_2$Y$_{122}$-$\beta_1$/F$_3$Y$_{731}$-$\alpha_2$ | 143       | 22%                            |
| E$_{1}$Q/F$_2$Y$_{122}$-$\beta_1$/F$_3$Y$_{731}$-$\alpha_2$ | 35        | 18%                            |
| E$_{1}$Q/F$_3$Y$_{122}$-$\beta_1$/F$_2$Y$_{731}$-$\alpha_2$ | 153       | 25%                            |

*Estimated error: ±5%; see Figure S18.

Table 1. Parameters Used for the ENDOR Simulations

3.3. $^{17}$O ENDOR with (E$_{12}$Q)-F$_3$Y$_{122}$-$\beta_1$/F$_3$Y$_{731}$-$\alpha_2$. An independent effort was made to obtain experimental evidence for a flipped Y$_{731}$ conformation in the trapped complex. We investigated whether a $^{17}$O ENDOR signal might be observable with a sample prepared using uniformly labeled $^{17}$O–Y$_{wt}$-$\alpha_2$ ($^{17}$O in the phenol groups). This experiment was motivated by our recent successful observation of a $^{17}$O ENDOR signal from water H-bonded to Y$_{356}$*. DFT calculations predicted a $^{17}$O–Y$_{731}$–Y$_{356}$* coupling of ~0.5 MHz for the flipped structure S5, slightly smaller than observed for H-bonded $^{17}$OH$_2$ (0.7 MHz) (Table S9). We further considered that this might make detection of this interaction more challenging. $^{17}$O has a lower gyromagnetic ratio than $^{19}$F ($\gamma(19F)/\gamma(17O) \approx 6.95$) and its quadrupolar coupling may lead to signal broadening. In addition, the $^{17}$O–Y$_{356}$* is only 35–40%-labeled based on the available $^{17}$O–Y used during expression (SI 6). A reference ENDOR signal, with a comparable concentration of predicted $^{17}$O spins in close proximity to Y$_{356}$* (i.e., 35–40 μM), is shown in Figure S15. Despite potential unexpected issues, we proceeded with the experiment as $^{17}$O should be a sensitive nucleus at short distances (<3 Å) and the $^{17}$O–Y$_{731}$ coupling for the stacked conformation should not be detectable, allowing us to test the flipped Y$_{731}$ model. As shown in SI 6.2, we were not able to observe any $^{17}$O couplings in three independently prepared samples. We have considered several possible explanations for these observations that may be related either to the experiment or to the use of F$_3$Y probes: (1) the $^{17}$O coupling might be smaller than the DFT prediction and not detectable; (2) F$_3$Y$_{731}$ could experience a different flipping ratio or rate of flipping relative to Y$_{731}$; (3) the F$_3$Y$_{122}$ used to initiate radical transfer in the experiment is likely reduced to its phenolate, not phenol as with Y$_{122}$*, and could play a role for the subunit interaction. These scenarios will be further discussed in the next section.

4. DISCUSSION

In this paper, we report the use of 94 GHz $^{19}$F ENDOR spectroscopy, which has provided new insight into the chemistry of RT between Y$_{356}$($\beta$) and Y$_{731}$($\alpha$) of E. coli RNR located at the subunit interface (Figure 2C,D). Success was possible using enzymes with site-specifically incorporated F$_3$Ys: F$_1$Y$_{122}$-$\beta_1$/F$_2$Y$_{731}$-$\alpha_2$ (or E$_{1}$Q/F$_2$Y$_{122}$-$\beta_1$/F$_3$Y$_{731}$-$\alpha_2$), when incubated with substrate (GDP) and effector (TTP), allowed trapping of the Y$_{356}$* pathway radical in an “active” $\alpha\beta$ complex during the reverse RT pathway process. PELDOR and HF-EPR analysis established the location of the trapped radical, and the double mutant provided a direct link to the recent cryo-EM structure. The studies allowed measurement of the $^{19}$F–Y$_{731}$ hyperfine couplings to Y$_{356}$*, which report on their interspin distances and provide interesting mechanistic implications.

Analysis of 94 GHz $^{19}$F ENDOR spectra of the Y$_{356}$* required careful evaluation and subtraction of $^{19}$F signals associated with unreduced F$_3$Y$_{122}$* and $^1$H backgrounds.
Nevertheless, comparison of the spectra acquired at 50 and 80 K allowed unambiguous assignment of three distinct couplings between F2Y731 and Y356.

Construction of small models of the three Ys and their DFT-predicted 19F HFC couplings, ENDOR orientation selection, and spectral simulations indicated that the 19F spectra are consistent with a mixture of flipped and stacked conformations of F2Y731 with respect to Y730 with flipped contributions of 25 ± 10% among the samples. While the flexibility of Y731 has been reported previously, the present results provide the first evidence for a conformation, in which the two pathway residues are located at an O–O distance of ∼3 Å, with potentially important consequences for understanding the interfacial PCET step. The presence of both conformations simultaneously suggests that they are energetically similar and may exist in equilibrium.

A number of different types of experiments have previously reported multiple Y731 conformations.26,30 In one study, in which CDP/ATP was incubated with wt-R411A-NH3Y731∗ α2 an NH3Y731∗ intermediate trapped in the forward RT was observed.26 The flipping was detected by PELDOR spectroscopy by its unusual Y356*/NH3Y731 distance. This distance, however, was only observed in conjunction with an additional mutation at α-R411A. This residue sits in the α/β interface. In addition, transient absorption experiments in solution using the same α-R411A mutation and a photo-oxidant indicated a kPCET between Y356-F-photoβ2 and Y731 much faster than dNDP formation, ~10 s⁻¹ versus 1–10 s⁻¹.30

On the other hand, neither in the cryo-EM structure with E52Q/F512β1 nor in the 15O ENDOR experiments, which both employed F512β1∗ and wt-α2, was the flipped conformation of Y731 observed. Thus, while the role of F512β1 in potentiating flipping is still unclear, the F512-phenolate generated at residue 122 during RT may not be the basis for a flipped Y731 conformation. In addition, the conditions for freeze-quenching the cryo-EM and ENDOR samples are very distinct in terms of protein concentration and glycerol content. A protein concentration of ∼80 µM had to be used for EPR samples, exceeding physiological RNR concentrations (ca. 1 µM). At elevated protein concentrations, the formation of αβ complexes has been reported.69,70 However, these complexes are incapable of producing Y356∗ and should not affect the analysis of EPR experiments, in which Y356∗ was observed selectively.

Overall, the complex interplay between Y356(β), Y731(α), R411(α), and other residues at the subunit interface is likely to be crucial for regulating the communication between the two redox-active Ys across the α/β interface.

Inspecting the predicted HFC parameters of the phenolic proton of F512Y731 with respect to Y356∗ is another interesting source of information. The DFT calculations predicted HFCs of ~6 MHz in models S4 and S5. It is important to rationalize this finding in the context of previous 1H ENDOR studies on H-bond interactions to Y356.27,28 In those studies, a 1H coupling in the range of 6 MHz was observed and assigned to one (or 2 equiv) H-bonded water molecule(s). The presence of the second water molecule was postulated to explain the unprecedented low gX value of Y356∗, i.e., 2.0062.27 The sharp peaks observed in our recent 263 GHz 15O ENDOR experiments support the presence of only a single water molecule.28 Given the similarity of coupling constants for the H-bonded protons for Y731 from either model S4 or S5, the flipped conformation provides an explanation for the 1H coupling consistent with these previous 1H ENDOR data. To date, however, no ENDOR study has provided information on the interplay between stacked/flipped Y731 and the water binding at Y356∗, which may be a key feature to control PCET across the interface. Interestingly, no distribution of gX values at Y356∗ is observed, indicating that the electrostatic environment is well defined and similar in both Y351 conformations. A mechanism, by which Y351 replaces a water molecule as a H-bond donor to Y356∗ upon flipping, could explain this finding.

4.1. Implication of Flipped Y731 in PCET across α/β. Observation of flipped F512Y731 in close distance to Y356, trapped in an active RNR complex, enables the examination of a mechanism for the PCET step between Y356∗ and Y731 for the first time.

The current hypothesis for interfacial PCET involving water, as noted above, was based on the ENDOR studies and the H-bond to Y356 assigned to water.74 Recent MD simulations27,28 based on the cryo-EM structure supported the role of water first suggested by Nick et al.57 The simulations additionally showed that water molecules can be present at the α/β interface including between Y356 and Y731 between Y356 and β-E52 (an interface residue), and support a pathway for water to escape to the bulk solvent.38,45 Interestingly, MD also revealed an equilibrium between flipped and stacked conformation for Y351, both populated at room temperature.45 Nevertheless, the reported flipped Y731 structure from the MD study still shows a long O–O distance to Y356 (∼8 Å on average), precluding a direct interaction between the two Ys.35

Thus, the mechanism of PCET between Y356 and Y731 (i.e., during reverse and forward RTs) remained to be resolved due to the long Y356–Y731 distance (∼8 Å) observed in the cryo-EM structure.38 We note that the published cryo-EM structure and ENDOR data have distinct problems. The resolution of the cryo-EM structure was insufficient to resolve waters. The ENDOR studies only detected water in the first coordination sphere of Y356∗, i.e., in a distance range of ∼3 Å.27,28

The 19F ENDOR data presented here, despite the issues raised, provide evidence for close interaction between the two Ys across the subunit interface in an active RNR construct. In our ENDOR-derived model S5, the O–O distance between Y356∗–Y731 amounts to 3.0 ± 0.2 Å, with a similar value in the related model S4. This distance is within the range of the distances reported for the pathway pair C439g−Y730 (O–S: 3.7 Å in the X-ray structure of α2 versus 3.4 Å in α-NH3Y730),13,44 as well as for the pair Y730−Y731 (O–O: 3.3 Å in α2 versus 2.7 Å in α-NH3Y730).13,44 For these pairs, independent quantum chemical calculations predicted a colinear PCET mechanism,24,71,72 in which the electron and proton are transferred individually in one step from the same donor to the same acceptor, although a water-assisted PCET has been proposed and discussed for the C439g−Y730 pair.73 Recently, also an alternative, glutamate (E623)-mediated proton transfer for the RT between Y731 and Y730 has been proposed based on MD simulations and QM/MM analysis.44 A key conclusion from the latter study based on the analysis of E623 was that forward and reverse RTs are different. Interestingly, our earlier large-scale DFT calculation on the pathway triad C439−Y740−Y731 predicted that the coordination of a water molecule to Y350∗ can stabilize this radical intermediate and the transition states to the next pathway intermediates, Y351 and C439g.24 Therefore, the calculation pointed to a functional role of water in PCET without its direct involvement as a proton donor or acceptor. Based on these considerations, we propose...
that our current results are consistent with a model of colinear PCET mechanism for the RT Y$_{756}$($\alpha$) $\rightarrow$ Y$_{731}$($\alpha$) $\rightarrow$ Y$_{730}$($\alpha$). This mechanism requires a conformational change of Y$_{731}$ during the long-range RT, as the next step (Y$_{731}$($\alpha$) $\rightarrow$ Y$_{730}$($\alpha$)) occurs in the stacked conformation of the Y$_{731}$/Y$_{730}$ pair.

5. CONCLUSIONS

Use of site-specifically incorporated unnatural amino acids and kinetic trapping in conjunction with high-field ENDOR, PELDOR, and EPR spectroscopies has given new insight into the PCET involving Y$_{756}$($\beta$) and Y$_{731}$($\alpha$) across the RNR subunit interface. $^{19}$F ENDOR revealed two sets of hyperfine coupling constants for F$_{2}$Y$_{731}$ caused by the occurrence of two distinct conformations. One set of hyperfine couplings is consistent with a stacked Y$_{731}$ conformation at $\sim$8 Å distance (O–O) to Y$_{756}$, as observed by cryo-EM. However, much larger $^{19}$F couplings revealed a second conformation, in which F$_{2}$Y$_{731}$ is flipped toward Y$_{756}$ at a much shorter O–O distance of $\sim$3 Å. This distance is similar to distances between other Y pairs on the RT pathway in $\alpha$, for which colinear PCET has been established.

These results reveal again the ability and importance of EPR spectroscopic methods and new experimental designs for the detection of multiple conformations in a biological machinery.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.2c02906.

Measurement of RNR activity, EPR sample preparation, EPR experiments (263, 34, and 94 GHz spectroscopies including relaxation measurements, radical yield determination, and background corrections), $^{17}$O ENDOR data, modeling of the tyrosine triad, and ENDOR simulations (PDF)

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Notes
The authors declare no competing financial interest.

Original spectroscopic data associated with Figures 5 and 9 as well as all xyz coordinates of the model structures can be accessed via the open database Göttingen Research Online (https://doi.org/10.26525/YXHC63).

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