Hydrogen Bond Network between Amino Acid Radical Intermediates on the Proton-Coupled Electron Transfer Pathway of E. coli α2 Ribonucleotide Reductase

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ABSTRACT: Ribonucleotide reductases (RNRs) catalyze the conversion of ribonucleotides to deoxyribonucleotides in all organisms. In all Class Ia RNRs, initiation of nucleotide diphosphate (NDP) reduction requires a reversible oxidation over 35 Å by a tyrosyl radical (Y122, Escherichia coli) in subunit β of a cysteine (C439) in the active site of subunit α. This radical transfer (RT) occurs by a specific pathway involving redox active tyrosines (Y132 ↔ Y156 in β to Y731 ↔ Y730 ↔ C439 in α); each oxidation necessitates loss of a proton coupled to loss of an electron (PCET). To study these steps, 3-aminotyrosine was site-specifically incorporated in place of Y731α and Y730α, and each protein was incubated with the appropriate second subunit β(α), CDP and effector ATP to trap an amino tyrosyl radical (NH2Y●) in the active α2β2 complex. High-frequency (263 GHz) pulse electron paramagnetic resonance (EPR) of the NH2Y●s reported the g values with unprecedented resolution and revealed strong electrostatic effects caused by the protein environment. 2H electron–nuclear double resonance (ENDOR) spectroscopy accompanied by quantum chemical calculations provided spectroscopic evidence for hydrogen bond interactions at the radical sites, i.e., two exchangeable H bonds to NH2Y731●, one to NH2Y730● and none to NH2Y356●. Similar experiments with double mutants α-NH2Y731/C439A and α-NH2Y731/Y730F allowed assignment of the H bonding partner(s) to a pathway residue(s) providing direct evidence for colinear PCET within α. The implications of these observations for the PCET process within α and at the interface are discussed.

INTRODUCTION

Ribonucleotide reductases (RNRs) catalyze the conversion of four nucleotides (CDP, UDP, ADP, GDP; NDPs) into the corresponding deoxynucleotides (dNDPs)1,2. Class Ia RNRs are found in nearly all eukaryotic and some prokaryotic organisms3 and are composed of two homodimeric subunits, α2 and β2, which form an active, transient α2β2 complex.4 Subunit α2 houses the catalytic substrate binding site and the binding sites for the allosteric effectors that govern specificity and activity of nucleotide diphosphate (NDP) reduction.5–7 Subunit β2 houses the diferrotyrosyl radical cofactor (the FeIII-Y●) essential for initiating NDP reduction. During each turnover, the FeIII-Y●/β2 oxidizes C439 in the active site of α2 where dNDP is produced, and then it is subsequently reoxidized. The oxidation occurs via a radical hopping mechanism over 35 Å between the two subunits along a specific pathway comprised of redox active amino acids (Y122 ↔ [W48?] ↔ Y156 in β2 ↔ Y731 ↔ Y730 ↔ C439 in α2). Reversible oxidation and reduction of Y during turnover requires release of the phenolic proton to an acceptor concomitant with the oxidation (Figure 1A), a mechanistic strategy to avoid formation of high-energy intermediates.8 While the long-range oxidation through aromatic amino acid residues in ribonucleotide reductase (RNR) is unprecedented in biology,4,8,9 proton-coupled electron transfer (PCET) mechanisms are involved in many fundamental processes in biology including photosynthesis, respiration and nitrogen fixation.10,11 RNRs can thus serve as a paradigm for understanding PCET in a complex biological machine and, in comparison with other systems, offer the opportunity to identify common principles that control this basic transformation. Since PCET is intrinsically of quantum mechanical nature because of both electron and proton tunnels, the large difference in mass (factor ~2000) causes the proton translocation to be limited to very short distances (<1 Å),8 while the electron may transfer over

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very long distances. Thus, in PCET the electron and the proton might be transferred to different acceptors (orthogonal or bidirectional) or they might move between the same donor/acceptor pairs (colinear). Description of the coordination of these events has been theoretically challenging and is summarized in recent reviews. 24,25 Investigation of the electron and proton pathways has required RNRs with site-specifically incorporated unnatural amino acids and electron paramagnetic resonance (ENDOR) combined with electron–nuclear double resonance (ENDOR) spectroscopies have provided a unique opportunity to access mechanistic details if the intermediate radicals can be detected.

The current model for this long-range oxidation is shown in Figure 1B. It was originally proposed based on the X-ray structures of α2 and β2 13-16 and a docking model of these subunits based on their shape complementarity, 15 and conserved residues. 17-20 Recent biophysical studies including pulsed electron–electron double resonance (PELDOR) studies 21,22 and small-angle X-ray scattering studies (SAXS) 23 and cryoEM 23 have established that the docking model is a reasonable representation of the “active” RNR structure in solution and that the oxidation occurs over >35 Å. Unfortunately there is little information about the molecular details of the interface between β and α, more specifically, the communication between Y356 in β2 and Y731 in α2. The last 25–30 amino acids of all β2 structures which include Y356 or its equivalent are structurally disordered and the last 15 amino acids of Escherichia coli β2 are largely responsible for the weak interaction between the subunits (Kd of ~0.1 to 0.2 μM) 17,24.

Studies of this unprecedented oxidation catalyzed by wt RNR have not been possible as protein conformational change(s) constitute the rate-limiting step(s) and kinetically mask all of the detailed chemistry of the radical transfer (RT) and the nucleotide reduction step. 26 However, use of technology in which the pathway tyrosines are replaced site-specifically with tyrosine analogues with altered pKas and reduction potentials, 4 and development of high frequency (HF) EPR 27,28 and ENDOR methods 25 to characterize Y• analogues generated with the mutant RNRs, are now unmasking the chemistry of this long-range oxidation. These studies taken together suggest that conformational gating is occurring within β and that radical intermediates formed at Y356, Y731, Y730, and C439 and substrate radical are likely in equilibrium along the pathway. 29,30 Their relative redox potentials are progressively uphill with corresponding lower concentrations. However, there is sufficient concentration of the most uphill radical (substrate radical) to drive the reaction to the right by a rapid, irreversible step proposed to be the loss of water during nucleotide reduction. Once the chemical reaction is complete, the reformation of the Y122• in β is energetically downhill. 4

The results from the incorporation of NH2Y site-specifically at 356–β, 731- and 730-α, the focus of this paper, have played a critical role in our current understanding of specific steps of the RT pathway and the conformational gating process. In all three cases, NH2Y•-β(α) is generated in ~30–40% yield upon incubation of the second subunit α(β), substrate CDP and allosteric effector ATP. 31,32 The formation of the NH2Y• is a biphase process with both phases kinetically competent in nucleotide reduction at 5 to 10% the rate of the wt-RNR. 31,32 Three recent experiments using this probe address the importance of PCET and the α2β2 conformational changes triggered by binding substrate and effector. Insight into the initial RT event within β has been studied by rapid freeze quench (RFQ) Mössbauer analysis using wt-β2/NH3Y730-α2/CDP/ATP. 33 The results established that the proton of the water bound to Fe1 (Figure 1B) is transferred to Y122• coupled with an electron, likely from Y356. Using the NH2Y730-α2 only the forward radical step is observed and interestingly the Fe1-OH now remains. These results support the model of orthogonal PCET in β2 and the exquisite control dependent on the proton’s location (Figure 1B).

Using the same experimental design, analysis of the NH2Y• by HF EPR and deuterium (2H) ENDOR provided the first spectroscopic insight suggesting the importance of colinear PCET within α2 and the structural relationship of Y731 and Y730 in the active α2β2 RNR complex. 25 The link between EPR data
in the active intermediate state and the X-ray structural data in the inactive state was supported by quantum chemical calculations of the energy optimized structural models of Y290•α and NH2Y290•α including 211 atoms (Figure 1C). The ENDOR analysis in conjunction with density functional theory (DFT) calculations revealed one strong (R O−O ~ 2.7 Å) and one weaker (R O−O ~ 3.4 Å) H bond proposed to be associated with residues adjacent to NH2Y290•α in the pathway, as well as another weak (R O−O ~ 3.0 Å) interaction with water. The water molecule was proposed to tune the RT rates in wt enzyme around Y290 by about 1 order of magnitude.25 Finally, our recent SAXS and pull-down experiments of RNR from this reaction mixture established that a single H atom transfer from NH2Y356 to Y122•α is sufficient to increase the weak α/β subunit affinity by a factor of 105.23 This result again highlights the importance and subtlety of conformational changes in the RT process.

In the current paper, experiments in which NH4Y has been site specifically incorporated at NH2Y356•β and at NH2Y730•α and NH2Y730•α (Figure 1C) in conjunction with HF EPR and HF 2H ENDOR spectroscopies, density functional theory (DFT) calculations using NH2Y730/731/α structures, have been employed in an effort to understand the electrostatic and H bonding environment at these residues in the RT pathway. HF EPR data were recorded at a microwave frequency of 263 GHz, which delivers unprecedented resolution in the spectra of the NH2Y•s. In addition, double mutants NH2Y730/C439A•α and NH2Y731/Y730F•β were prepared and the 2H ENDOR results compared to the single mutant results in an effort to assign the source of the H bonds observed to specific residues. Our results support the colinear concerted PCET model within α and provide spectroscopic evidence for the unusual stacking of Y730/Y731 in the active α2β2 complex.

RESULTS

Electrostatics and Hydrogen Bond Interactions around the Radical Intermediates. To probe the electrostatic environment of the radical intermediates at the three different positions in the RT pathway we examined the HF EPR spectra formed after incubation of β-NH2Y356•α-NH2Y730•α-NH2Y730 with their respective complementary wt subunits, either α or β, CDP, and ATP (Methods). Figure 2 presents 263-GHz pulse EPR spectra of the individually trapped NH2Y•s recorded with a prototype quasi-optical EPR spectrometer (Methods). The buffer was exchanged with D2O to enhance EPR resolution. At this frequency, all gα, values of NH2Y•s (ND•Y•) are clearly resolved and shifted by about 1 ppt (or 0.001) from the value calculated for a free NH2Y• (gα free = 2.0061). This shift is significant, on the order of the effect predicted from several hydrogen bonds.25,34,35 gα, values were calibrated with the spectrum of the stable radical Y122•α in β2, which is present in all samples and visible only at low temperatures. Additionally, gα, slightly decreases starting from the most buried intermediate NH2Y730•α (gα = 2.0054) to NH2Y356•α (gα = 2.0049) located at the subunit interface. As a control, spectra were also recorded in protonated buffer and the g values were best reproduced (Supporting Information, Figure S1). Simulations of the 263 GHz spectra combined with 94 GHz spectra (the latter frequency was used to constrain the simulation, Figure S2) led to a consistent set of g values and C−β proton hyperfine (hf) couplings (see chemical structure in Figure 1 inset) that are summarized in Table 1. Thus, the EPR spectra of the single mutants in the active enzyme are

![Figure 2. 263-GHz pulse EPR spectra of different NH2Y•s intermediates.](image)

**Figure 2.** 263-GHz pulse EPR spectra of different NH2Y•s intermediates. Electron spin−echo (ESE) detected spectra of intermediates without (top spectrum for NH2Y356•α/NH2Y730, 10 K) and with relaxation filtering (bottom spectra for NH2Y730•α, 70 K). The spin−echo sequence used to suppress the signal of Y122•α (box) is based on differences in the transverse relaxation times (T2) of the radicals: NH2Y•s and Y122•α. At T = 70 K, the signal associated with the Y122•α decays during the acquisition (box, purple dotted line) and does not contribute to the spin echo signal. ESE detected EPR spectra of the NH2Y• radicals in H/D exchanged buffer: ND•Y730•α (black), ND•Y730•α (red) and ND•Y731•α (blue). Experimental conditions: ESE (π/2−τ−π−echo) spectra: π/2 = 60−110 ns, τ = 290 ns, 250−500 averages/point, acquisition time/spectrum = 1.5−3 days, T = 10 and 70 K.

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Table 2. Summary of g Values and C−β hf Couplings of NH2Y• at Residues 730, 731, 356

The values were obtained from combined simulations of the 263 and 94 GHz spectra and compared with those obtained from DFT calculations. The 14N hyperfine tensor of the NH2Y• was not varied in the simulations and kept Aα = 2.4 MHz, Aβ = 1.6−5.5 MHz, and Aγ = 30.7 MHz.25,26 Hf values are in MHz. Uncertainty in g values is about 0.05 ppt for the experiments and 0.5 ppt for DFT calculations. Uncertainty in hf couplings is up to 10% from spectral simulations and up to 20% in DFT calculations. Value reported in ref 28. Value from 2-amino-4-methylphenol radical.25

contributed by a radical species (per mutant) with a well-defined microenvironment (g value) and molecular orientation. This finding underlines the importance of a specific structural arrangement in the active state to permit radical propagation.
The observed shift of \( g_e \) values is a hallmark for a substantial effect of either positive charges and/or hydrogen bond interactions. Since hydrogen bonds are expected to have a predominant effect on \( g \) values, a possible correlation of the observed \( g_e \) shifts with the number and strength of hydrogen bonds was examined.

**Hydrogen Bonds between On-Pathway Amino Acids.** We probed exchangeable hydrogen bonds around the trapped NH$_2$Y· intermediates by ENDOR spectroscopy at 94 GHz. ENDOR reveals the spectrum of magnetic nuclei that are in the coordination sphere (usually \( \leq 5 \) Å) of the observed radical. After buffer exchange, exchangeable protons are substituted by deuterons (abbreviated D, with the nucleus denoted \(^2\)H), which become visible by ENDOR in the \(^2\)H resonance region. Figure 3 illustrates the \(^2\)H Mims ENDOR spectra of the three NH$_2$Y· intermediates (ND$_2$Y·) trapped under comparable conditions as in the EPR experiments. The spectrum of ND$_2$Y$^{730}$· was initially reported in ref 25, and it is included here for further analysis. All three spectra contain a broad, almost featureless background extending over \( \pm 2 \) MHz, which arises from the strongly coupled amino deuterons. Additionally, two samples, ND$_2$Y$^{730}$· and ND$_2$Y$^{731}$·, show pairs of sharp peaks in the region \( \leq 1 \) MHz that is usually dominated by deuterons in the hydrogen bond range (Figure 3A, peaks marked in red and blue). The spectra of ND$_2$Y$^{730}$· and ND$_2$Y$^{731}$· look quite similar, with the peaks from ND$_2$Y$^{731}$· being slightly shifted to larger couplings. The sharp peaks are split by an additional small coupling, i.e., the quadrupolar coupling. The ENDOR spectrum can also detect weak couplings (\( r_0 \sim 2.1 \) Å) if they can be resolved from matrix deuterons (matrix line). Indeed, additional small differences are observed here also in the central resonance region (\( \pm 0.3 \) MHz). In ND$_2$Y$^{730}$·, this region was proposed to be associated with a water molecule (wat1, Figure 1) conserved in the structure of wt \( \alpha^2_{15,16,37} \) and ND$_2$Y$^{730}$·\( \alpha^2_{32} \). This resonance pattern is now absent in ND$_2$Y$^{731}$· and replaced by a matrix line. Interestingly the spectrum of ND$_2$Y$^{730}$· lacks any sharp peaks and is associated with resonances of the amino deuterons and a matrix line.

**H-Bond to NH$_2$Y$^{731}$·.** Further experimental evidence for the hydrogen bond at NH$_2$Y$^{731}$· was derived from orientation selective HF \(^2\)H ENDOR spectra recorded at different field positions in the EPR line. Figure 4A shows Mims ENDOR spectra recorded at the canonical orientations \( B_0 \parallel g_y, g_x, g_z \) within the region of \( \pm 1.5 \) MHz. Powder patterns are still observed at orientations \( B_0 \parallel g_y \) and \( g_x \) as orientation selection is moderate for the large excitation bandwidth of the pulses (\( \sim 1.8 \) mT) as compared to the total EPR line width (\( \approx 8 \) mT); however, clear differences in the line shapes are visible. The smallest hf coupling, taken as the center of the sharp peak, is observed at \( B_0 \parallel g_z \) and the hf tensor displays a form \( |A_z| \geq |A_x| > |A_y| \) (using the definition \( |A_j| = A_j \leq A_j < A_j \)). Previous DFT calculations have indicated that such a tensor form is typical for a deuteron directed almost perpendicular to the tyrosine ring plane, as previously reported also for ND$_2$Y$^{730}$·\( \alpha^2_{25} \) and having the smallest component along the H bond. Such a tensor reflects couplings that still contain some contribution from a scalar interaction arising from orbital overlap. In the following, we define hydrogen bonds with these tensor properties as strong to moderate (\( r_0 \sim 1.7-2.0 \) Å). The definition used here is consistent with ref 10 but expanded by the definition of moderate bonds. Considering that the \( g_z \) tensor component lies along the C-O bond and \( g_y \) points to the side of the amino group, \( \alpha^2_{25} \) the Euler angles \( \alpha, \beta, \gamma \) between hyperfine A and \( g \) tensor obtained from the simulation (Table 2) are consistent with a hydrogen bond directed almost perpendicular to the plane of the NH$_3$Y$^{731}$·. The mutual tensor orientation is illustrated in Figure 4, inset. Simulations of the entire \(^2\)H ENDOR spectrum with the parameters obtained here additionally indicate that the spectrum of a ND$_2$Y$^{731}$· can be well reproduced by the contribution of a single hydrogen bond (Figure 3A) according to the observed intensity ratio of the sharp peaks with respect to the amino deuterons.

**Preparation and Characterization of NH$_2$Y$^{731}$/Y$^{730}$F- and NH$_2$Y$^{730}$/C$_{439}$A-\( \alpha^2 \)2.** In an effort to assign the H bonding interactions observed with NH$_2$Y$^{731}$· and NH$_2$Y$^{730}$·\( \alpha^2 \) by \(^2\)H ENDOR, double mutants NH$_2$Y$^{731}$/Y$^{730}$F and NH$_2$Y$^{730}$/C$_{439}$A were generated, in which one of the proposed H bonds was removed. The proteins were expressed, purified to homogeneity and characterized by stopped flow-Vis spectroscopy monitoring the rate of loss of Y$^{732}$F (\( 410 \) nm) and rate of formation of the NH$_3$Y· (320 or 325 nm) in the presence of wt-\( \beta^2 \)/CDP/ATP, Figure S3. The results are summarized in
In the case of both double mutants, the altered kinetics of GHz EPR spectroscopy in samples frozen at 30 s (Figure S4). unsuccessful. Both double mutants were also characterized by 9 GHz-ENDOR spectra and orientation of the 2H hf tensor does not display any hydrogen bonds but only a matrix line, as also discussed in ref 2S. The inset shows the orientation of the 2H hf tensor from the simulation.

Figure 4. 2H-ENDOR spectra and orientation of the hydrogen bond at NH2Y731•. 2H 94-GHz Mims ENDOR spectra (black lines) were recorded at field positions in the EPR line parallel to the canonical orientations of the g tensor, i.e., for $B_0 \parallel g_x, g_y, g_z$. Simulations of the spectra (red dashed-dotted curves) were performed as described in the Methods section. Contributions from the hydrogen bond are shown additionally as red peaks. The obtained values are reported in Table 2. A line broadening of 50 kHz was used. Experimental parameters: $\pi/2 = 20$ ns, $\tau = 320$ ns, RF pulse length = 40 $\mu$s, shot repetition time = 150 ms, acquisition time = 50 h/spectrum, random RF acquisition, T = 10 K. The low S/N required operation at very low T and contribution of Y122• could not be separated here. However, Y122• does not display any hydrogen bonds but only a matrix line, as also discussed in ref 2S. The inset shows the orientation of the 2H hf tensor from the simulation.

Table S1 and compared with results from similar experiments on the single mutants. With NH2Y731/Y730F-α2, NH2Y• was formed with biphase kinetics resulting in 34 ± 3% conversion, similar to the amount formed with NH2Y731α2 (32 ± 3%). The kinetics of both phases, however, were slower: 1.5 ± 0.1 and 0.3 ± 0.03 s$^{-1}$ compared to 9.6 ± 0.6 and 0.8 ± 0.1 s$^{-1}$. Similar studies with NH2Y730/C439α-α2 resulted in formation of NH2Y• in only 14 ± 1% conversion, compared with 39% in the single mutant. Furthermore, the rate constant for its formation was decreased ~10 fold for the double mutant: from 12 ± 1 to 0.13 ± 0.01 s$^{-1}$ and only a single kinetic phase was measured. Attempts to express NH2Y730/C439S-α2, unfortunately, were unsuccessful. Both double mutants were also characterized by 9 GHz EPR spectroscopy in samples frozen at 30 s (Figure S4). In the case of both double mutants, the altered kinetics of pathway radical formation and its altered amplitude in the case of NH2Y730/C439α-α2 are likely reflective of the importance of these H bonding interactions in tuning of PCET within α2. To test for possible conformational differences between the NH2Y• intermediates in the double vs the single mutants, we measured their inter spin distances to the diagonalized Y122• by PELDOR spectroscopy (Figure S5 and S6). The experiments revealed distances of 3.8 and 3.9 nm, respectively, consistent within error (±1 Å) with the distances observed in the single mutants and from the α2/α2 docking model in wt; however, the assignment of the 3.9 nm distance in the mutant NH2Y730/C439A is more uncertain due to the low radical yield and S/N ratio of the PELDOR traces. HF EPR spectra (263 GHz) of NH2Y• trapped in the double mutants also revealed $g_s$ values (Figure S7) different from those in the single mutants, i.e., shifted by +0.3–0.4 ppt in the direction of a free NH2Y• as expected after removal of a H bond (Table 1).

DFT Optimized Structures of α-NH2Y731•. To obtain a model structure of the radical localized at NH2Y731•, DFT calculations on representative model systems for NH2Y731• were performed and the obtained magnetic parameters for $g_x$ and for H bonds were compared with the experimental values. As a starting point, we used the DFT energy optimized structure of Y731• that was previously reported (Methods). Three large models (up to 216 atoms, Figure S8) were considered, which differed by the inclusion of zero, one or two water molecules. The models have taken into account all residues in an interaction sphere of about 5 Å around the oxygen of Y731. The obtained energy-minimized structures are represented in Figure 5.

We found that the presence or absence of the water molecules has an impact on some residues arrangement around NH2Y731• but not on the formation of a strong H bond with Y730•. In optimized models 1 and 2, the distance from the oxygen of NH2Y731• to the hydrogen of the phenol group of Y730 is 1.7 Å. In absence of wat1 (model 2), R411 approaches NH2Y731• with hydrogens from its guanidinium group at distances of 2.1 and 2.6 Å from the oxygen of NH2Y731•. The calculated $g_s$ values from the two models differ by about 0.5 ppt, with the $g_s$ values of model 2 being closer to the experimental ones (Table 1). In model 3 a second water molecule (wat2) was included as observed in some X-ray structures of wt-α and NH2Y731/750-α (Figure S9) in the vicinity (R3-O• ~ 2.6–3.6 Å) of residue 731. The optimized structure of model 3 finds wat2 within hydrogen bond distance (R3-O• ~ 1.9 Å) to NH2Y731•; however, a stronger H bond to the phenoxyl hydrogen of Y730 remains (R3-O• ~1.6 Å). The $g_s$ value for the model 3 structure is consistent with the experimental value (Table 1). The computed EPR parameters for the H bond distances and orientations relative to Y730 are listed in Table 2.
Table 2. Summary of EPR Parameters for the Hydrogen Bond to NH2Y731$^\bullet$

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<td>−1.7</td>
<td>−2.0</td>
<td>−163</td>
<td>122</td>
<td>81</td>
<td>−0.04</td>
<td>−0.06</td>
<td>0.09</td>
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<tr>
<td>ND$_2$ D(1)</td>
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<td>−2.6</td>
<td>−3.4</td>
<td>−98</td>
<td>99</td>
<td>72</td>
<td>−0.05</td>
<td>−0.06</td>
<td>0.11</td>
</tr>
<tr>
<td>ND$_2$ D(2)</td>
<td>0.67</td>
<td>−2.63</td>
<td>−3.97</td>
<td>−100</td>
<td>84</td>
<td>−45</td>
<td>−0.05</td>
<td>−0.06</td>
<td>0.11</td>
</tr>
</tbody>
</table>

$^a$Parameters were obtained from simulations of the orientation selective 94-GHz ENDOR spectra and comparison with the DFT models. The signs of the couplings from the simulation are only relative to each other within one tensor. The Euler angles ($\alpha, \beta, \gamma$) are defined from the A or Q to the g tensor based on the y convention (positive sign for a rotation is counterclockwise, second rotation is around the y axis). The A and Q (quadrupole) tensor are chosen such that $|A_x| < |A_y| < |A_z|$. Within this definition, for both the amino deuterons and the H bond deuteron the $A_x$ direction results along the bond direction. Euler angles from DFT (in the ORCA output positive rotations are defined clockwise) were transformed into the magnetic resonance convention, for comparison. Uncertainty in the parameters from the DFT and ENDOR simulations is estimated up to about 20%.

Figure 5. DFT optimized structures of NH$_2$Y$_{731}$.$^\bullet$. Left: Model 1 includes the water molecule wat1. Center: Model 2 has no water molecules. Model 3 contains a second water molecule wat2, which is observed in some X-ray structures (see text). Residues in interaction distance are in gold. Distances are given in Ängström.

Particularly, all models display an orientation of the strong H bond to NH$_2$Y$_{731}$.$^\bullet$ provided by Y$_{730}$ which is compatible with the ENDOR data within an uncertainty of about 20% (Table 2, Figure S10).

Considering the accuracy of the calculations, on the order of 0.5 ppt, all three models give $g_x$ values in principle compatible with the experiment. However, considering that the error in the trend of the calculation is smaller than 0.5 ppt, model 2 and 3 better reproduce the experimental g values. The results indicate that the strong $g_x$ shift at NH$_2$Y$_{731}$.$^\bullet$ can be contributed by the combined effect of a strong H bond to Y$_{730}$ and the interaction either to a water molecule (model 3) or alternatively to the positively charged arginine R$_{411}$ (model 2). The findings are further supported by calculations on small model systems (Figure S11), in which the effect of the individual residues R$_{411}$ or Y$_{730}$ was systematically tested. Both scenarios represented in models 2 and 3 find some precedents in the literature. Studies on π–cation interactions revealed that these are common between amino acids like arginine and aromatic amino acids like tyrosine.$^{39,40}$ Other studies on small peptides showed that tyrosine/arginine interactions can alter the reduction potential of tyrosines.$^{41}$ On the other hand, in PS II two water molecules have been proposed to interact with the redox active $Y_x$ during PCET and affect their g values.$^{34}$ We note that protons of
either wat2 or R411 possibly located at distances $\geq 1.9$ Å from the phenoxy oxygen might not be resolved in an ENDOR spectrum. Finally we note that the $g$ values for these NH$_2$Y$\bullet$ can also be affected by the $\beta$ subunit, which cannot be modeled at present.

## DISCUSSION
The electronic and structural features of the NH$_2$Y$\bullet$s trapped in the RT pathway of E. coli RNR report on how the local protein environment has reorganized after a PCET step to stabilize these intermediates. At each radical state, the protein has rearranged to accommodate a released electron and a proton, a scenario that can be probed by two EPR parameters: the shift in the $g_z$ value caused by positive charges and the hyperfine interaction of protons forming hydrogen bonds of varying strengths. The first parameter is directly accessible from high-frequency EPR spectra but contains only indirect information on hydrogen bond interactions. The second parameter, i.e., the hyperfine coupling to protons exchanged by deuterons, requires ENDOR spectroscopy and much more extensive analysis, which in turn, when attainable, uniquely delivers a high resolution structure of the H bond partners. In this study we report both parameters for the NH$_2$Y$\bullet$s trapped within the RT pathway of RNR $\alpha$ subunit. All $g_z$ values of the NH$_2$Y$\bullet$s in the three pathway positions are similar and show a considerable shift from the calculated value for a free NH$_2$Y$\bullet$ (Table 1), consistent with a strongly perturbed electrostatic environment. However, our data reveal that the number, orientation and strength of exchangeable hydrogen bonds at these residues are intrinsically different, reflecting the subtle difference in protein architecture and giving direct insight into the hydrogen bond network involved in PCET.

The ENDOR data display one strong hydrogen bond and one moderate H bond with NH$_2$Y$_{356}\bullet$ and one strong hydrogen bond with NH$_2$Y$_{731}\bullet$. In both cases, the hyperfine couplings and tensor orientations of the two strong bonds are very similar (Tables 2 and S2) and indicate that the hydrogen must reside between the two tyrosyl rings (Figures 3 and 4). The direction of the hydrogen bond to NH$_2$Y$_{731}\bullet$ extracted from the present hyperfine and quadrupole tensors in the ENDOR data (Figure 4) are consistent with the directions predicted by the DFT calculations for a bond with Y$_{730}$ (Figure 5) no matter the number of water molecules considered in the calculation. Moreover, the ENDOR peaks (Figure 3B) of this strong bond disappear when Y$_{730}$ is mutated to F$_{730}$. These results taken together provide strong evidence for a direct hydrogen bond from Y$_{730}$ to NH$_2$Y$_{731}\bullet$ and strongly suggest that the proton is directly transferred via colinear PCET, as was proposed by previous quantum chemical calculations. Thus, the results support the current model for PCET (Figure 1), that is, that Y$_{731}\bullet$ functions as a proton acceptor for the subsequent RT step to Y$_{730}$. Moreover, the present DFT calculations provide further insight into this PCET step. They reveal that in each case, the H bond must be directed almost perpendicular to the tyrosyl $\pi$ system to reproduce the size of the hyperfine tensors and the $g_z$ shift of each NH$_2$Y$\bullet$. The results together provide strong support for the $\pi$ stacking of Y$_{731}$/Y$_{730}$ in the “active” $\alpha2/\beta2$ complex. It should be pointed out that this stacking is observed in some, but not all of the “resting”$\alpha2$ structures (yeast$^{31}$ and NH$_2$Y structures$^{32}$ show residue 731 in multiple conformations) and that currently no atomic resolution structural data are available for the complex. The unusual Y/ Y $\pi$ stacking and the implications of this design relative to other configurations for optimum efficiency of transfer has been studied by theorists.$^{33,46}$ The work of Kaila and Hummer$^{45}$ indicates that this configuration results in strong electronic coupling and adiabatic, colinear PCET, consistent with our data.

Finally, the X-ray structure of the resting states of the wt, NH$_2$Y$_{730}\bullet$ and NH$_2$Y$_{731}$−$\alpha$ and our previous DFT optimized structure of NH$_2$Y$_{730}$− and Y$_{730}$−$\alpha$ show that residue C$_{439}$ is in hydrogen bond distance to Y$_{730}$ (Figure 1C). The present experiments with the double mutant NH$_2$Y$_{356}$−/ C$_{439}A$ corroborate the assignment of C$_{439}$ as a second moderate hydrogen bond partner of Y$_{730}$−. Thus, the most direct interpretation of our data combined with the calculated reduction potentials in ref 25. is that residue Y$_{730}$− acts as the direct hydrogen bond acceptor for C$_{439}$−. In principle it still remains possible that water does provide the H-bond, and that the C$_{439}A$ mutant disrupts the H-bonding pattern and eliminates it; however, our experimental data provide no evidence for the participation of a water in this proton transfer step, in contrast to a recent proposal of Bu et al. This group using DFT and QM/MM calculations on several model systems, in which Cys could transfer a proton to Y$\bullet$ including one with the “real protein” environment for C$_{439}$ Y$_{730}$−, suggested that the conserved water molecule (H$_2$O$_{138}$ in their notation is wat1 here) moves and inserts itself between these residues promoting a double proton-coupled electron transfer step. However, their calculated barrier for direct PCET between C$_{439}$ and Y$_{730}$− of 60 kcal/mol is inconsistent with ours and Siegbahn and co-workers$^{45}$ calculations, which delivered a barrier of 8–9 kcal/mol. Intriguingly, in one of their model systems with a Cys and Tyr located on different peptide chains$^{47}$ mimicking the RNR configuration, the barrier for PCET is almost identical (9 kcal/mol) to our data in the real system.

The studies with NH$_2$Y$_{731}$−/Y$_{730}$F (Figure 3B) failed to reveal a H bonding interaction with a second proton as observed with NH$_2$Y$_{730}$−/HSC$_{439}$A. However, in the center of the 3H-ENDOR spectrum, the broad matrix line could be indeed contributed by one or several weak H bonds, which are not resolved. Nevertheless, the DFT calculation revealed that R$_{411}$ is capable of rearranging to form weak hydrogen bonds (2.1 Å) if it is not sterically hindered by water molecules, and that this configuration, along with the strong H bond to Y$_{730}$ can reproduce the observed $g_z$ value. However, a model with two waters (Figure S), also is capable of recreating the experimentally measured $g_z$ value. The absence of structural insight about the $\alpha/\beta$ subunit interface and the ability to use the protein environment (R$_{411}$ or two waters) to subtly alter the electrostatic environment, suggests multiple factors contribute to $g_z$ in addition to the strong H bond and our methods cannot currently distinguish between them.

Perhaps the most unexpected observation from the current studies are the results with NH$_2$Y$_{356}$−$\alpha$2. In contrast with the pathway residues in $\alpha$2, no exchangeable moderate H bonds to NH$_2$Y$_{356}$− are observed outside the matrix line, yet the $g_z$ value is perturbed to a similar extent as those for the NH$_2$Y$\bullet$s in $\alpha2$. Our inability to observe crystallographically Y$_{356}$ within $\beta2$ alone or in $\alpha2/\beta2$ prevents any specific conclusions about the origin of the $g_z$ shift. However, the data still contribute to our understanding of PCET at this position. For example, one possible mode of communication between Y$_{356}$ and Y$_{731}$ could involve $\pi$ stacking similar to that observed between Y$_{731}$ and Y$_{730}$. This type of interaction is unlikely, however, given the
absence of any moderate H bond. A second possible model of communication for PCET between Y356 and Y731, could involve a water molecule or network of water molecules at the subunit interface. The amino protons of NH$_2$Y$_{731}$• would likely obscure these weak H bonds as they are adjacent to the matrix line, precluding their detection. The greatly perturbed $g_z$ value of NH$_2$Y$_{731}$• in the absence of strong or moderate H bonds requires an altered electrostatic environment provided by the protein. Whether this environment could be provided by water requires an altered electrostatic environment provided by the interface48

In conclusion, the present data establish a hydrogen bond network between residues 731–730 in the α2 subunit. The observed hydrogen bonds and directions provide very strong support for a colinear PCET mechanism, consistent with the recent finding that the turnover rate constants within α are very fast, >14000 s$^{-1}$, when conformational gating from β is removed by photoinitiation. These results differ dramatically from the PCET process at the α/β interface and within β. Our data indicate that colinear PCET and π stacking between Y$_{356}$ and Y$_{731}$ are unlikely, and also reveal the importance of the electrostatic protein environment. Additional spectroscopic experiments could be informative, but structural insight is also essential. Within α2 the combination of protein engineering, spectroscopic data, and quantum chemical calculations has provided much insight into the PCET process within this subunit. Nature appears to have utilized multiple PCET strategies to achieve this long-range oxidation over 35 Å.

■ METHODS

Sample Preparation. 4-(2-Hydroxyethyl)-1-piperazinethanesulfonic acid (Hepes) was purchased from EMD Bioscience. Adenosine-5′-triphosphate (ATP), cytidine-5′-diphosphate (CDP), reduced β-nicotinamide adenine dinucleotide phosphate (NADPH), hydroxyurea (HU), kanamycin (Km), chloramphenicol (Cm), 2X YT media, M9 minimal Salts, l-arabinose (ara), β-mercaptoethanol (β-ME), streptomycin sulfate and NH$_2$Y$_7$ were purchased from Sigma-Aldrich. Isopropyl-α-D-thiogalactopyranoside (IPTG) and 1,4-dithiothreitol (DTT) were purchased from Promega. Tris-(2-carboxyethyl)phosphate (TCEP) hydrochloride was purchased from Thermo Scientific.

Nucleotide primers were purchased from Invitrogen and Pfu Ultra II polymerase was purchased from Stratagene.

\[ (\text{His})_3\text{-wt-α2 \ (2200 \text{nmol/min/mg)} \text{ and wt-β2 \ (7000 \text{nmol/min/mg) and 1.2 Y} \text{• β2 were expressed and purified by standard protocols}. \]

All α2 mutants were prereduced with 30 mM DTT and 15 mM HU before use. E. coli thioredoxin (TR, 40 U/mg) and thioredoxin reductase (TRR, 1800 U/mg) used in assays were isolated according to manufacturer's protocol to generate each mutant. The Quikchange kit (Stratagene) was used according to manufacturer's protocol to generate both subunits.

Site-Directed Mutagenesis to Generate pET-nrdA Mutants


<table>
<thead>
<tr>
<th>Mutant</th>
<th>Primer 1</th>
<th>Primer 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAT$<em>{370}$ → TTT$</em>{370}$</td>
<td>G TTC AAA ACA C TG</td>
<td>TTT TAG CAG AAC ACC CG</td>
</tr>
<tr>
<td>TTG$<em>{439}$ → GCC$</em>{439}$</td>
<td>GCT GAG TCT ACG CTT GCC GAG</td>
<td>GCC GAG CAG GAG ATC</td>
</tr>
<tr>
<td>TGG$<em>{439}$ → AGC$</em>{439}$</td>
<td>GCT GAG TCT ACG CTT GCC GAG</td>
<td>GCC GAG CAG GAG ATC</td>
</tr>
</tbody>
</table>

mM Tris, 5% glycerol, 1 mM PMSF, pH 7.6) contained 1 mM TCEP. The typical yield of purified protein is ~6–7 mg/g cell paste.

**Samples for High Field EPR and ENDOR.** α-NH$_2$Y$_7$, α-NH$_2$Y$_7$β, NH$_2$Y$_{356}$, and their double mutants were combined with the corresponding wt(β/α) 1:1 at final complex concentrations of 100–200 μM in D$_2$O and H$_2$O assay buffer as previously described. The reaction was initiated at 25 °C by adding CDP and ATP with final concentrations of 2 and 6 mM, respectively, and manually freeze quenched after 10–20 s inside the EPR tube with liquid N$_2$.

**High-Frequency Pulsed EPR and ENDOR.** Echo-detected EPR spectra at 263 GHz were recorded on a Bruker Elexys E780 quasi optical spectrometer using a single mode (T(E01)) cylindrical resonator (Bruker BioSpin) with a typical quality factor of 500–1000. Maximum microwave power coupled to the resonator was about 15 mW. The electron spin echo (π/2−τ−π−τ−echo) was recorded with π/2 pulse lengths of 60–100 ns. The EPR spectrum of Y$_{123}$ in wt/β was used as a reference (Figure S1) at 250 μM concentration in assay buffer to calibrate magnetic field. Samples for 263 GHz EPR were inserted in capillaries (0.33 OD, Vitrocom CV20335/Q) in typical volumes of ~50 nl. Samples for 94 GHz spectroscopy contained typical volumes of 2 μl in 0.9 mm OD capillaries. Samples frozen in liquid nitrogen were loaded into the resonator immersed in liquid nitrogen and then transferred into the precooled EPR cryostat.

94 GHz pulse EPR and ENDOR spectra were recorded on a Bruker E680 W-band spectrometer with 400 mW microwave output power (Bruker Power Upgrade 2). 94 GHz $^3$H Mims ENDOR spectra were simulated using EasySpin "pepper"-routine running under MATLAB. The parameters were set to the experimental conditions. The line width was set to 3 G with a line broadening contribution of a 1:1 Gaussian to Lorentzian.

**Simulations of ENDOR Spectra.** Spectra were processed by phasing and baseline correction. Derivatives were obtained by fitting every four points with a second order polynomial and differentiating the function in MATLAB (version 7.10). ENDOR spectra were simulated using EasySpin "salt"-routine using perturbation theory. $^3$H EPR spectra were simulated using EasySpin "pepper"-routine running under MATLAB. The parameters were set to the experimental conditions. The line width was set to 3 G with a line broadening contribution of a 1:1 Gaussian to Lorentzian.

**DFT Calculations.** All calculations have been performed with the ORCA 3.0.0 program package. The initial model structures were based on the large models 7 and 8 used in ref 25 augmented by the amino group at Y$_{371}$. Geometry optimizations have been performed using the B3P86 gradient corrected density functional $^{63,64}$ in combination with Ahlrichs’ TZVP basis set of triple-$\zeta$ quality. $^{65,66}$ Grimme’s dispersion correction $^{67,68}$ has been applied on top of the SCF calculation. The Resolution of the Identity approximation with the corresponding auxiliary basis sets has been employed throughout. Cartesian constraints were imposed on the position C$_{\alpha}$ of Y$_{730}$ Y$_{371}$ and C$_{439}$ as well as C$_{\alpha}$ and C$_{\beta}$ of all surrounding residues. Additionally
the Cartesian coordinates of the hydrogen atoms in the truncated GPD model replacing the bonds between C4 and C5 of the ribose as well as the bond between C1 of the ribose and the base were kept fixed. The g-values were calculated using the NH2Tyr-Ct as gauge origin.

The EPR calculations and geometry optimization of the small models were carried out with the B3LYP64,69,70 hybrid density functional in combination with the RIJCOSX71 approximation. Here only the dihedral angle of the peptide bond of Y730 and Y731 was fixed and the Cartesian restraints for all surrounding Cα’s were kept. In the small models a solvation model (COSMO72) with polarity ε = 24 was used to compensate protein in

***ASSOCIATED CONTENT***

Supporting Information

Simulations of the 263 and 94 GHz EPR spectra (1−2). Characterization of the double mutants (Y730NH2Y/Y730Fα2 and Y730NH2/ C730Aα2) by stopped-flow Vis, 9 GHz EPR, PELDOR and HF EPR measurements (3−7). Full size of the large DFT models (8), crystal structures of α displaying water molecules (9), hf tensor orientation from ENDOR simulation and DFT models (10) and small DFT models (11). Coordinates and absolute energies of the DFT optimized models of Figure 5. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

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