Radical transfer in *E. coli* ribonucleotide reductase: a NH$_2$Y$_{731}$/R$_{411}$A-α mutant unmask a new conformation of the pathway residue 731†

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Ribonucleotide reductases (RNRs) catalyze the conversion of ribonucleotides to deoxyribonucleotides in all living organisms. The catalytic cycle of *E. coli* RNR involves a long-range proton-coupled electron transfer (PCET) from a tyrosyl radical (Y$_{122}$) in subunit β to a cysteine (C$_{439}$) in the active site of subunit α, which subsequently initiates nucleotide reduction. This oxidation occurs over 35 Å and involves a specific pathway of redox active amino acids (Y$_{122}$ ↔ [W$_{48}$?] ↔ Y$_{356}$ in β2 to Y$_{731}$ ↔ Y$_{730}$ ↔ C$_{439}$ in α2). The mechanisms of the PCET steps at the interface of the α2β2 complex remain puzzling due to a lack of structural information for this region. Recently, DFT calculations on the 3-aminotyrosyl radical (NH$_2$Y$_{731}$)-α2 trapped by incubation of NH$_2$Y$_{731}$/α2/β2/CDP(substrate)/ATP(allosteric effector) suggested that R$_{411}$-α2, a residue close to the α2β2 interface, interacts with NH$_2$Y$_{731}$ and accounts in part for its perturbed EPR parameters. To examine its role, we further modified NH$_2$Y$_{731}$/α2 with a R$_{411}$A substitution. NH$_2$Y$_{731}$/R$_{411}$A generated upon incubation of NH$_2$Y$_{731}$/R$_{411}$A-α2/β2/CDP/ATP was investigated using multiphoton excitation electron double resonance (PELDOR) and electron–nuclear double resonance (ENDOR) spectroscopies. The data indicate a large conformational change in NH$_2$Y$_{731}$/R$_{411}$A relative to the NH$_2$Y$_{731}$ single mutant. Particularly, the inter-spin distance from NH$_2$Y$_{731}$/R$_{411}$A in one αβ pair to Y$_{122}$ in a second αβ pair decreases by 3 Å in the presence of the R$_{411}$A mutation. This is the first experimental evidence for the flexibility of pathway residue Y$_{731}$-α2 in an α2β2 complex and suggests a role for R$_{411}$ in the stacked Y$_{731}$/Y$_{730}$ conformation involved in collinear PCET. Furthermore, NH$_2$Y$_{731}$/R$_{411}$A serves as a probe of the PCET process across the subunit interface.

**Introduction**

Coupling of electron and proton transfers between donors and acceptors in proteins is ubiquitous in biology and can occur in a stepwise or concerted fashion. The concerted case avoids high energy intermediates and is designated as proton coupled electron transfer (PCET).¹ The mechanisms of these couplings are fundamental to our understanding of photosynthesis, respiration, synthesis of DNA building blocks, and many other processes. Unresolved issues describing these mechanisms have been articulated in several recent comprehensive reviews, with different mechanisms dictated by transfer distances, protein environment and dynamics.²,³ When the proton and electron donor and acceptor are distinct, the mechanism involves orthogonal PCET; when the donor and acceptor are the same, it involves collinear PCET.⁴,⁵ A different mechanism in which a proton is transferred through water chains over long distances in concert with electron transfer (ET) has also been recently studied and discussed extensively in model systems.⁶,⁷ In all mechanistic cases, since the electrons and protons have very different masses, electrons tunnel over large distances (10–15 Å) while proton tunnelling is restricted to shorter distances, on the order of hydrogen bond lengths.⁸,⁹ This distance dependence complicates the issue of proton management. One important representative of the diversity of PCET mechanisms in proteins is found in the class I ribonucleotide reductases (RNRs). These enzymes catalyze the conversion of nucleotides to deoxynucleotides, the monomeric precursors required for DNA replication and repair in all eukaryotic and some prokaryotic organisms.¹⁰,¹¹ In this paper, we use the *Escherichia coli* (*E. coli*) class Ia RNR as a model system to interrogate the PCET process across the interface of the two subunits of this enzyme, proposed to involve two redox active protein tyrosine residues, one on each subunit, and a water interface between the subunits.¹²

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The *E. coli* RNR consists of two homodimeric subunits, α2 and β2.1-3 The enzyme is active when a transient β2β2 complex is formed.3 α2 contains the active site for nucleotide reduction and two allosteric effector binding sites that regulate the specificity and the rate of reduction.15-19 β2 harbors the essential di-iron tyrosyl radical cofactor (Fe(II)-Y122)20,21 During each turnover, Y122-β2 oxidizes C439→α2 to a thyl radical, which subsequently initiates dNDP production.11 There are X-ray structures of the individual subunits, and a docking model of the α2β2 complex places Y122-α2 at a distance of about 35 Å from C439.20,23 These initial studies led to the first formulation of radical transfer (RT) in RNR via a radical hopping mechanism involving a pathway of conserved amino acids (Y122 → [W48] → Y356 in β2 to Y351 → Y730 → C439 in α2). Biochemical24 and biophysical (EPR,25,26 SAXS,27 and cryoEM28) studies confirmed that the docking model provides a reasonable representation of *E. coli* RNR in its transient, active form and led to a detailed mechanism of RT over such a long distance.4,12,24 Nevertheless, in wild-type (wt) *E. coli* RNR, the rate limiting step, conformational change(s) upon substrate and allosteric effector binding to α2, has prevented spectroscopic detection of any intermediates in this process.7 The recent development of methods to site-specifically incorporate tyrosine analogs with altered pKₐ and reduction potentials has permitted the detection of pathway radical intermediates29-32 and, combined with state-of-the-art EPR spectroscopy,12,28,31,34 has started to reveal the molecular basis of the long-range RT in RNR.14

These experiments have led to the current model illustrated in Fig. 1, which involves orthogonal PCET35 steps within subunit β2 and collinear PCET steps within the α2 subunit.13,14 However, the mechanism of the PCET process at the subunit interface between Y356 in β2 and Y731 in α2 remains elusive, as structural information on the C-terminal 35 amino acids of β2, including a putative proton acceptor E350 and Y356 (Fig. 1), is missing.32

Our recent high-field (HF) EPR/ENDOR and DFT investigations using the 3-aminotyrosine mutants NH₂Y₇₃₁-α2 and NH₂Y₇₃₁-α2, which generate the corresponding NH₂Y⁻ upon incubation with β2, CDP (substrate) and ATP (allosteric effector), established that an unusual stacked conformation of residues 730 and 731, observed in some X-ray structures of α2 (ref. 23 and 36) (see ESI, Fig. S1†), occurs in the α2β2 complex.12,28 However, the X-ray structure of NH₂Y₇₃₀-α2 (PDB 2XO4) alone exhibited multiple conformations for Y₇₃₁-α2, with one rotated away from NH₂Y₇₃₀-α2 toward the α2β2 subunit interface.39 This “flipped” conformation was accompanied by reorientations of R₄₁₁ and N₇₃₁ in α2. Further comparison of NH₂Y₇₃₀-α2, NH₂Y₇₃₁-α2 and NH₂Y₇₃₆-β2 by HF EPR indicated that the electrostatic environment of all three transient NH₂Y’s is strongly perturbed and that their hydrogen bond interactions are intrinsically different.12,31 Interestingly, one of our DFT models of the protein environment for NH₂Y₇₃₁-α2 required R₄₁₁-α2 to explain the perturbed gₓ value observed and suggested that R₄₁₁-α2 approaches to NH₂Y₇₃₁-α2 within 2.6 Å (Fig. S1†,12 Therefore, to examine the role of R₄₁₁-α2 during the PCET process in *E. coli* RNR, we generated two mutants: R₄₁₁A-α2 and the double mutant NH₂Y₇₃₁/R₄₁₁A-α2. Here, we report the incucation of NH₂Y₇₃₁/R₄₁₁A-α2 with β2/CDP and ATP, which generates the NH₂Y₇₃₁/R₄₁₁A-α₂β₂ complex. Using advanced EPR methods, including 263 GHz pulse EPR and 34 GHz PELDOR/DEER (pulsed electron–electron double resonance) and ENDOR (electron–nuclear double resonance) spectroscopies, we have provided evidence for a new conformation of NH₂Y₇₃₁/R₄₁₁ that is “flipped” towards the subunit interface in the α2β₂ complex. This is the first time an alternative conformation of any pathway tyrosine (NH₂Y₇₃₁\') has been observed and it provides a new probe of the PCET mechanism across the subunit interface, which remains unknown.

**Experimental**

**Materials**

4-(2-Hydroxyethyl)-1-piperazineethanesulfonic 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), 2XYT media, M9 Minimal Salts, D-arabinose (ara), β-mercaptoethanol (β-ME), streptomycin sulfate and NH₂Y were purchased from Sigma-Aldrich. Isopropyl-β-D-thiogalactopyranoside (IPTG) and 1,4-dithiothreitol (DTT) were purchased from Promega. Tris(2-carboxyethyl)phosphine (TCEP) hydrochloride was purchased from Thermo Scientific. Nucleotide primers were purchased from Invitrogen, and Pfu Ultra II polymerase was purchased from Stratagene.

**Site-directed mutagenesis to generate R₄₁₁A-α2 and NH₂Y₇₃₁/R₄₁₁A-α2**

The Quikchange kit (Stratagene) was used to generate each mutant according to the manufacturer’s protocol. The templates pET28a-nrdA and pET28a-nrdA Y₇₃₁Z were amplified with primer 5’-G CAG GAA CTT GGC TCT ACC GGT GCG ATC TAT ATT CAG AAC GTT GAC-3’ and its reverse complement.
and used to insert a GCG (Ala) at position 411. The sequences were confirmed by QuintaraBio Laboratory. All constructs contain an N-terminal (His)_6-tag with a 10 amino acid linker.  

Expression, purification and activity assays of R_{411}A-α2 and NH_{2}Y_{731}/R_{411}A-α2

(His)_6-wt-α2 (2750 nmol min⁻¹ mg⁻¹) and wt-β2 (7000 nmol min⁻¹ mg⁻¹), and 1.2 Y/β2 were expressed and purified by standard protocols.  

Expression and purification of R_{411}A-α2 and NH_{2}Y_{731}/R_{411}A-α2 followed previous protocols, except that the purification buffer (50 mM Tris, 5% glycerol, 1 mM PMSF, pH 7.6) for NH_{2}Y_{731}/R_{411}A-α2 contained 1 mM TCEP. The yields of purified R_{411}A-α2 and NH_{2}Y_{731}/R_{411}A-α2 were 10–12 mg g⁻¹ and 6–7 mg g⁻¹ cell paste, respectively. The activity of R_{411}A-α2 (0.2 μM) and NH_{2}Y_{731}/R_{411}A-α2 (1 μM) was determined in the presence of 50-fold excess of wt-β2 with 3 mM ATP, 1 mM [^3]H-CTP (4850 cpm nmol⁻¹), 30 μM TR, 0.5 μM TRR, and 1 mM NADPH in assay buffer (30 mM HEPES, 1 mM EDTA, 15 mM MgSO₄, pH 7.6). The amount of CTP was determined by the method of Steeper and Steuart.  

For single turnover experiments, NH_{2}Y_{731}/R_{411}A-α2 (5 μM) was incubated with wt-β2 (5 μM), 3 mM ATP, and 1 mM [^3]H-CTP (20 000 cpm nmol⁻¹) in assay buffer. The dissociation constant (K_d) for R_{411}A-α2 and wt-β2 was determined in H₂O and D₂O buffers by the competitive inhibition assay  

Samples for HF EPR and PELDOR spectroscopy

NH_{2}Y_{731}/R_{411}A-α2 and wt-β2 were mixed 1:1 to a final concentration of 160–180 μM in D₂O assay buffer as previously described. These protein concentrations resulted in >95% binding between subunits. The reaction was initiated at room temperature by adding CTP and ATP to final concentrations of 1 and 3 mM, respectively. The reactions were manually freezecquenched in liquid N₂ within 10–23 s. The PELDOR sample was prepared by adding glycerol (OD)₃ to a final concentration of 10% (v/v) 16 s after the initiation of the reaction. This reaction was manually freeze-quenched after 56 s as just described. The NH_{2}Y_{731}^+ accounted for 30–33% of the total spin for all the samples used in this work, which was similar to the yields reported previously.  

HF pulsed EPR spectroscopy

Echo-detected (ESE: π/2 – τ – π – echo) EPR spectra at 263 GHz were recorded on a Bruker Elexys E870 quasi optical spectrometer using a single mode (7E011) cylindrical resonator (9501610 – Bruker BioSpin) with a typical quality factor of 500–1000. The maximum microwave power coupled to the resonator was about 15 mW. Samples for 263 GHz EPR were inserted in capillaries (0.33 mm OD, Vitrocom CV20335) with typical volumes of ca. 50 nL. 94 GHz ESE spectra were recorded on a Bruker E680 spectrometer with a 400 mW W-band power setup (Bruker power upgrade – 2). Samples for 94 GHz ESE contained typical volumes of 2 μL in 0.84 mm OD capillaries (Wilmad S6X84). All manually freeze-quenched samples were immersed in liquid N₂ and loaded into pre-cooled EPR cryostats.

34 GHz PELDOR spectroscopy

34 GHz ESE and PELDOR spectra were recorded on a Bruker E580 X/Q-band spectrometer equipped with a Bruker EN 5107D2 pulse EPR/ENDOR resonator. The spectrometer was power-upgraded with a Q-band TWT amplifier, providing about 170 W output power at 34.1 GHz. PELDOR experiments were recorded with an overcoupled resonator. The center of the mode was chosen for the pump frequency for measurements at 20 K. However, for measurements at 50 K the detection frequency was set in the center of the cavity mode to enhance detection sensitivity. Q-band samples contained typical volumes of 10 μL in 1.6 mm OD capillaries (Wilmad 222T-RB).

Processing and simulation of EPR spectra

Spectra were processed by baseline correction. Derivatives of the absorption spectra were obtained by fitting every four points with a second order polynomial and differentiating the function in MATLAB. EPR spectra were simulated using the EasySpin-4.5.5 “pepper”-routine which was run in MATLAB.

DFT calculations

DFT calculations were performed with the ORCA 3.0.0 program package. The geometry optimization of the neutral NH₂Y⁻ was performed using the unrestricted B3LYP hybrid density functional in combination with the def2-TZVPP basis set and def2-TZVPP/JK auxiliary basis set. To take into account the electrostatic environment of the radical intermediate at the protein interface, a solvation model (COSMO) with the polarity of ethanol (ε = 24) was used. Otherwise, Grimme’s dispersion corrections and RIJCOSX approximations were employed. The energy converged to 10⁻⁶ Eh, the hyperfine couplings and g values were calculated using NH₂Y⁻/C₄ as the gauge origin. The def2-TZVPP basis set was consistent with the geometry optimization step. The C2–C1–C8–Cz dihedral angle of the NH₂Y⁻ was changed stepwise with a geometry optimization for each step. The xyz coordinates for one of the optimized models are given in the ESL.

PyMOL models

The docking model refers to the αβ2 complex structure generated from the individual wt-α2 and wt-β2 X-ray structures. In order to predict distances, the mutant E. coli RNR structure (PDB 2XO4) was overlaid with the wt-α2 structure in the docking model using PyMOL, which first performs a sequence alignment and then aligns the structures to minimize the root mean square deviation between the structures.
Results and discussion

Preparation and characterization of R411A-a2, NH2Y731/R411A-a2 and ND2Y731/R411A-a2

Our recent studies on NH2Y731-a2 (ref. 12) suggested that R411 might interact with NH2Y731, partially accounting for the measured EPR and ENDOR parameters. To investigate this proposal, R411A-a2 was generated and characterized. Because the mutation is proposed to be at the interface of a2 and β2, the dissociation constant (Kd) for subunit interactions was also examined and was determined to be 0.94 ± 0.33 μM (Fig. S2A †). Under these conditions, this mutant was shown to have a specific activity of 467 ± 22 mmol min⁻¹ mg⁻¹, 17% of that of the wt enzyme (2750 mmol min⁻¹ mg⁻¹). The reduced activity and weaker subunit binding suggest that R411 plays a functional role.

Furthermore, we characterized the role of R411 in the oxida-
tion of Y122 to a double exponential with

$$k_{\text{fast}} = 3.6 ± 0.5 \text{s}^{-1} \text{(amplitude 8%)}$$

and

$$k_{\text{slow}} = 0.47 ± 0.03 \text{s}^{-1} \text{(amplitude 21%)}$$

(Table S1). The rate constants for NH2Y731'a2 in the single mutant control were similar: $k_{\text{fast}} = 9.6 ± 0.6 \text{s}^{-1}$ and $k_{\text{slow}} = 0.8 ± 0.1 \text{s}^{-1}$. However, in this case, the fast phase accounted for 27% and the slow phase accounted for 13% of the NH2Y731'a2. The biphase kinetics of NH2Y731'a2 formation in both cases is attributed to multiple conformations that give rise to NH2Y731'a2. From 25 s to 100 s, NH2Y731 in the double mutant reaction disappeared with a $k_{\text{obs}}$ of 0.02 ± 0.003 s⁻¹, while with the single mutant, disappearance occurred with a $k_{\text{obs}}$ of 0.005 ± 0.002 s⁻¹. Analysis of the Y122'-β disappearance kinetics was unsuccessful at early time points due to the detection limits, as described in SI-4.

Given the distinct kinetics of our double mutant relative to the NH2Y731-a2, the 9 GHz EPR spectrum of the sample generated from the reaction of NH2Y731/R411A-a2 with wt-β2, ATP, and CDP quenched after 25 s was recorded and is shown in Fig. S5A and C. The subsequent to subtraction of Y122, 32% of the total spin is associated with NH2Y731/R411A-a2 with no spin loss. This result is similar to that of the single mutant, NH2Y731'a2. A comparison of their spectra, as shown in Fig. S5B †, revealed substantial differences in their hyperfine interactions, suggesting that further characterization of this radical might provide insight into the function of R411. Therefore, the role of R411 in the RT pathway was further studied with advanced EPR spectroscopy.

HF EPR of ND2Y731/R411A-a2

To examine the generated ND2Y731/R411A-a2, we took advantage of the proximity of Y122 to the di-iron cluster and its altered relaxation properties. Pulsed EPR spectra of ND2Y731/R411A-a2 at 34, 94 and 263 GHz were recorded in D2O buffer at 70 K and are shown in Fig. 2A. The use of D2O considerably simplifies the EPR spectra due to the absence of 1H hyperfine (hf) splittings arising from the amino protons. The ND2Y731/R411A-a2 EPR spectrum at 34 GHz is mainly dominated by the large hf couplings with the deuterons of the amino group and the two Cβ-methylene protons.44 On the other hand, the 94 and 263 GHz EPR spectra are dominated by g-anisotropy, and the relative contributions of $g$- and hf-anisotropy are strongly dependent on the operating magnetic field. The $g$ values of ND2Y731/R411A-a2 are best resolved at 263 GHz and are consistent with the values from our previous ND2Y studies.12,34 The 94 GHz spectra reveal differences in the hf splitting of the Cβ-methylene protons (Fig. 2A, marked with an arrow): the large hf splitting of the Cβ-methylene proton visible in the central line of ND2Y731-a2 (red) is missing in ND2Y731/R411A-a2 (black). This splitting is also absent in the 263 GHz spectrum. The EPR spectra were simulated iteratively to find a global solution for the contributing hf couplings. All of the EPR data and simulations, in which the previously reported44 hf coupling for 14N is used, are consistent with the NH2Y731 generated in the NH2Y731/R411A-a2/β2 complex being a single, well-oriented radical species with one set of magnetic parameters, which are listed in Table 1 (see also Fig. S7 †). This finding is not self-evident, as our previous experiments with other double mutants, NH2Y731/Y339F-a2 and NH2Y731/C419A-a2, showed distributions in $g$ values indicative of multiple radical environments and/or molecular orientations.12

Interestingly, we do not observe changes in the $g$ values between ND2Y731/R411A-a2 and ND2Y731-a2. This is unexpected because the $g_2$ value is affected by the electrostatic environment of a radical,47 and the R411A mutation has changed the local environment of ND2Y731'a2, as demonstrated by the substantial changes in the Cβ-methylene 1H couplings (Table 1). These couplings are related to the dihedral angle $\theta_{CB}$ between the Cβ-H bond and the p$_z$ orbital axis of C1 (Fig. 2B), and therefore provide information on the molecular orientation of the tyrosyl and 3-aminotyrosyl radicals.44 The dihedral angle can be extracted from the McConnell equation ($a_{iso(C-H)} = B_1 \times P_{CI} \times \cos^2 \theta_{CB}$)48 which provides a semi-empirical relationship for the observed isotropic constant $a_{iso}$. The C2-C1-Cβ-Cz angle of

References

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ND$_2$Y$_{731}$'/R$_{411}$A-$\alpha$2 is estimated to be $\approx$ 90° by using $B_1$ of 162 MHz (ref. 59) for tyrosyl radicals, an electron spin density $\rho_{C1}$ of 0.214,12 and an isotropic Cβ-methylene proton hf coupling $a_{iso} = 10 \pm 1$ MHz (Table 1). This dihedral angle is indeed consistent with the hf couplings of the two Cβ-methylene $^1$H resonances being indistinguishable, as reported in Table 1 and seen in Fig. 2B and C. This result was confirmed by DFT calculations on the observed hf couplings of NH$_2$Y', in which the ring orientation was modeled with respect to the backbone and showed a symmetric orientation relative to the $p_z$ orbital axis of C$_1$ (Fig. 2B). In this calculation, a $\theta_{C\beta}$ angle of 90° corresponds to $a_{iso} = 9 \pm 3$ MHz (grey area in Fig. 2C) for both Cβ-methylene protons, H$_{B2/1}$.

### ENDOR for detection of hydrogen bonds to ND$_2$Y$_{731}$'/R$_{411}$A-$\alpha$2

Given that the R$_{411}$A mutation had little effect on $g_s$, $^2$H ENDOR spectroscopy was used to further examine a possible correlation of the observed $g_s$ value ($g_s = 2.0051$) with the hydrogen bonding environment. Fig. 3 illustrates the $^2$H Mims ENDOR spectra of ND$_2$Y$_{731}$'-$\alpha$2 and ND$_2$Y$_{731}$'/R$_{411}$A-$\alpha$2. Both spectra contain a broad signal that extends over $\pm 2$ MHz, arising from the strongly coupled amino deuterons, which is a common feature of ND$_2$Y' Mims ENDOR spectra.13,33 However, we observe that the $^2$H hf tensor previously assigned to the moderately strong hydrogen bond between Y$_{730}$ and Y$_{731}$ in ND$_2$Y$_{731}$'-$\alpha$2, which is almost perpendicular to the tyrosine ring plane,12 is absent in the ND$_2$Y$_{731}$'/R$_{411}$A-$\alpha$2 spectrum. Therefore, the hydrogen bonding environment of NH$_2$Y$_{731}$'/R$_{411}$A-$\alpha$2 is distinct from that of the single mutant, consistent with the different side chain conformations observed by HF EPR spectroscopy. Note that almost the complete EPR line of ND$_2$Y$_{731}$'/R$_{411}$A-$\alpha$2 can be excited at 34 GHz by using very short microwave pulses.
and thus hf couplings cannot be missed due to orientation selective effects.

Although no exchangeable moderately strong hydrogen bonds (r₀-H = 1.7–2 Å) to ND₂Y₇₃₁/R₄₁₁A-α₂ are observed, the ENDOR spectra of ND₂Y₇₃₁/R₄₁₁A-α₂ are exhibited a broad and structured matrix line, which is associated with weak hf interactions of the radical with distant nuclei (see Fig. 3, inset). The structure in this matrix line suggests the presence of weakly coupled deuterons that cannot be resolved from the matrix ones (matrix line). We note that the ENDOR spectrum of ND₂Y₇₃₁/R₄₁₁A-α₂ is reminiscent of the one previously observed for ND₂Y₃₅₆-β₂, also located at the subunit interface and likely surrounded by a defined hydrogen bonded network of water molecules.⁴ The similarity between the ENDOR spectra of ND₂Y₃₅₆-β₂ and ND₂Y₇₃₁/R₄₁₁A-α₂ suggests a similar origin for the gₑ values in these two mutants, which is distinct from that in ND₂Y₇₃₁-α₂. As noted above, in the case of ND₂Y₃₅₆-β₂ the gₑ value was also strongly shifted (NH₃Y₃₅₆: gₑ = 2.0049 vs. free NH₃Y; gₑ = 2.0061 (ref. 33)). Therefore, we propose that the gₑ shift in NH₃Y₃₅₆/R₄₁₁A-α₂, as well as in ND₂Y₃₅₆-β₂, arises from weakly coupled hydrogen bonds observed in the 0.3 MHz region of the ENDOR spectrum. The complexity of the g tensor interpretation was underlined by our recent DFT calculations, in which three distinct models for NH₃Y₇₃₁-α₂ resulted in similar g-shifts.⁵ Overall, these data clearly indicate that the molecular orientation of ND₂Y₇₃₁/R₄₁₁A-α₂ is different to that of ND₂Y₇₃₁-α₂ and is affected by R₄₁₁A-α₂ substitution.

**PELDOR gives evidence for a conformational change in ND₂Y₇₃₁/R₄₁₁A-α₂**

Our previous PELDOR studies⁶ have demonstrated that half sites reactivity of *E. coli* RNR allows for the detection of the diagonal inter-spin distance between Y₁₂₂ in one αβ pair and any radical trapped in the second αβ pair (Fig. 4A).⁶,⁶² To gain insight into the location of NH₃Y₇₃₁/R₄₁₁A-α₂, three sets of PELDOR experiments were recorded using broadband excitation with a high-power Q-band set up at different excitation positions in the EPR line⁶¹–⁶⁶ (see Fig. 4B and S8†). The recorded time traces are displayed in Fig. 4C and show substantial differences in modulation depth (10 to 50%), which is typical for orientation selection effects. Trace D₁ also shows a higher frequency component that arises from the parallel component of a dipolar Pake pattern (Fig. S8†). For this reason, the background corrected PELDOR time traces from the three sets of experiments were summed and the resulting trace was analyzed as shown in Fig. 4C and D. Additional comparison of the Fourier-transformed traces (Fig. S8†) shows that the sum trace leads to an almost complete Pake pattern. Distance distribution analysis revealed a clear dominant peak at 35 Å with a distance distribution of Δr = ±2.7 Å. We note that the error in the peak distance is much less than the distribution and is estimated to be ±0.5 Å. The width of the distance distribution is slightly larger than in previous measurements within the *E. coli* RNR αββ₂ complex,⁵⁵,⁶³,⁶⁶ suggesting more conformational heterogeneity for ND₂Y₇₃₁/R₄₁₁A-α₂, consistent with the observed flexibility of this residue. Nevertheless, the results clearly indicate that the R₄₁₁ mutation induces a conformational change of ND₂Y₇₃₁-α₂ into a new well-defined conformation.

The peak distance of 35.0 Å has never been observed between any radicals formed in this pathway before, and it is 3 Å shorter than that previously measured for ND₂Y₇₃₁-α₂.⁶ This distance might appear to be rather close to the initial distance (prior to turnover) between the two stable Y₁₂₂’s, that is 33.1 ± 0.2 Å.⁶ To confirm our assignment, we recorded PELDOR experiments at higher temperature (50 K), in which the Y₁₂₂-β₂ contribution to the re-focused echo is filtered and ND₂Y₇₃₁-α₂ is the only radical species detected (Fig. S9†). However, Y₁₂₂-β₂ can still be excited by the pump pulse and contributes to the PELDOR signal. Under these conditions, any distance observed in the PELDOR experiments at 50 K is related to Y₁₂₂-ND₂Y₇₃₁ and cannot be associated with the Y₁₂₂-Y₁₂₂ distance, as the latter radical is not detected. The distance distribution analysis of the 50 K measurements yielded a peak distance of 35.3 Å with a distribution of Δr = ±2.0 Å, and thus validated our assignment (see Fig. S9†).

To gain more insight into the conformational change of NH₃Y₇₃₁/R₄₁₁A-α₂ and the role of R₄₁₁, we examined the available X-ray structures of *E. coli* α₂’s in the R₄₁₁ region. In the structure of *E. coli* NH₃Y₇₃₀-α₂ (2XO4),⁸ Y₇₃₁ is flipped away from NH₃Y₂₃₀ as shown in Fig. 5. This altered conformation is compared with a second α in the unit cell, in which the Y₇₃₁ is not flipped. To match the 35 Å distance observed by PELDOR spectroscopy, the aromatic ring of NH₃Y₇₃₁ must rotate away from Y₂₃₀ toward the β₂ subunit, as observed for Y₇₃₁ in the *E. coli* Y₇₃₀NH₃Y-α₂ structure (Fig. 5).
This reorientation is also supported by the ENDOR data, which indicate that the stacked conformation between NH$_2$Y$_{731}^\cdot$ and Y$_{730}$ with a shared, perpendicular hydrogen bond is absent in NH$_2$Y$_{731}^\cdot$/R$_{411}$A-$\alpha_2$, and that the radical is instead surrounded by weakly coupled hydrogen bonds, likely water molecules at the $\alpha_2/b_2$ subunit interface. The exposure of NH$_2$Y$_{731}^\cdot$/R$_{411}$A-$\alpha_2$ to the interface and the buffer in this new conformation might be the origin of the instability of the radical as compared to the single mutant (Table S1†). We have also examined another possible conformation, in which the amino group of NH$_2$Y$_{731}$-$\alpha_2$ moves to occupy the vacancy created by the mutation of arginine to alanine. This conformation is displayed in Fig. S10.† However, in this case the expected distance between the oxygen atoms of NH$_2$Y$_{731}$ and Y$_{122}$ exceeds the observed distance by $\approx$2 Å. We note that the “flipped” conformation has not been observed in the single mutant NH$_2$Y$_{731}^\cdot$/R$_{411}$A-$\alpha_2$ or in the double mutant NH$_2$Y$_{731}^\cdot$/Y$_{730}$F-$\alpha_2$, in which Y$_{731}$ lacks its hydrogen bonding partner, suggesting the importance of R$_{411}$ in stabilizing the stacked conformation. This change between a flipped and non-flipped conformation of the interface Y might play an active role in the PCET process between Y$_{731}$ and Y$_{356}$ in wt RNR, the mechanism of which is still not understood. With the wt enzyme, this conformational change is kinetically masked by physical gating, which rate-limits RNR, and is too fast to be detected based on the recently
measured rate constants for electron transfer (ET) (10^4 to 10^5 s^-1) at the interface by photo-RNRs that unmask this gating.68.69 Thus, the R411A mutation might have fortuitously allowed detection of this movement at the subunit interface.

While the lack of structural information at the subunit interface poses a challenge for a mechanistic understanding of interfacial PCET, the detection of the NH2Y731/R411 provides us with a spectroscopic probe of this interface. Mutagenesis and site-specific isotopic labeling of interface residues could provide us with additional insight into how this step is controlled. Finally, the mechanism of PCET across the subunit interface observed with the E. coli RNR is likely to be conserved in all class I RNRs based on their subunit structures and the conserved weak subunit associations dictated by the C-terminal tail of β2.70,71 The pathway for oxidation is conserved between RNR classes Ia, Ib and Ic, as is the regulation of the pathway by NDP/ effector binding.72 Thus, while the "details" of the radical transfer mechanism might be different in the individual class I RNRs, general principles will likely emerge from the studies on E. coli RNR, given all of the evolutionarily conserved features.

Conclusions

This study has revealed that the E. coli RNR double mutant NH2Y731/R411A-a2 unmasks a new conformation of pathway residue 731 in the α2β2 complex. This is the first experimental evidence for the flexibility of this pathway or any pathway residue in the active enzyme. The results have provided insight into the mechanisms of PCET within α2, as well as through the α2β2 interface. First, R411 appears to play a role in the stabilization of the stacked conformation of Y356 and Y731, and thus in the facilitation of collarine PCET within the α2 subunit. Second, the new conformation is consistent with Y731 pointing toward the subunit interface, in the direction of the adjacent pathway residue Y356, located in the flexible C-terminal tail of subunit β2. The flexibility of these two contiguous pathway residues, which have been suggested to communicate during PCET,69 might be the key to driving the RT chemistry at the subunit interface through water clusters.57 This opens up a new hypothesis for the PCET mechanism between residues Y356-α2 and Y731-β2, which could involve a gated conformational change in Y731-α2 in wt RNR on a fast time scale, not observable without the R411A mutation. While this hypothesis remains to be proven, the present results will serve as a basis to design new experiments aimed at detecting a possible combined role of Y351-α2 and Y356-β2 in PCET through the subunit surface.

Acknowledgements

We acknowledge Igor Tkach for the help with technical aspects of the HF EPR spectrometers. MK thanks Karin Halbmair for the assistance with PELDOR measurements. We gratefully acknowledge financial support for this work from Deutsche Forschungsgemeinschaft DFG-IRTG 1422 (GRK 1422 to MK and MB) and DFG-SPP 1601, the Max Planck Society and NIH (GM29595 to JS).

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