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Radical transfer in *E. coli* ribonucleotide reductase: a NH₂Y₇₃₁/R₄₁₁A- α mutant unmasks 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 (Y₁₂₂[•]) in subunit β 2 to a cysteine (C₄₃₉) in the active site of subunit α 2, which subsequently initiates nucleotide reduction. This oxidation occurs over 35 Å and involves a specific pathway of redox active amino acids (Y₁₂₂ $\leftrightarrow$ [W₄₈?] $\leftrightarrow$ Y₃₅₆ in β 2 to Y₇₃₁ $\leftrightarrow$ Y₇₃₀ $\leftrightarrow$ C₄₃₉ in α 2). The mechanisms of the PCET steps at the interface of the α 2 β 2 complex remain puzzling due to a lack of structural information for this region. Recently, DFT calculations on the 3-aminotyrosyl radical (NH₂Y₇₃₁[•])- α 2 trapped by incubation of NH₂Y₇₃₁- α 2/ β 2/CDP(substrate)/ATP(allosteric effector) suggested that R₄₁₁- α 2, a residue close to the α 2 β 2 interface, interacts with NH₂Y₇₃₁[•] and accounts in part for its perturbed EPR parameters. To examine its role, we further modified NH₂Y₇₃₁- α 2 with a R₄₁₁A substitution. NH₂Y₇₃₁[•]/R₄₁₁A generated upon incubation of NH₂Y₇₃₁/R₄₁₁A- α 2/ β 2/CDP/ATP was investigated using multi-frequency (34, 94 and 263 GHz) EPR, 34 GHz pulsed electron–electron double resonance (PELDOR) and electron–nuclear double resonance (ENDOR) spectroscopies. The data indicate a large conformational change in NH₂Y₇₃₁[•]/R₄₁₁A relative to the NH₂Y₇₃₁[•] single mutant. Particularly, the inter-spin distance from NH₂Y₇₃₁[•]/R₄₁₁A in one $\alpha\beta$ pair to Y₁₂₂[•] in a second $\alpha\beta$ pair decreases by 3 Å in the presence of the R₄₁₁A mutation. This is the first experimental evidence for the flexibility of pathway residue Y₇₃₁- α 2 in an α 2 β 2 complex and suggests a role for R₄₁₁ in the stacked Y₇₃₁/Y₇₃₀ conformation involved in collinear PCET. Furthermore, NH₂Y₇₃₁[•]/R₄₁₁A serves as a probe of the PCET process across the subunit interface.

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Introduction

Coupling of electron and proton transfers between donors and acceptors in proteins are 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.^{1,8,9} 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, $\alpha 2$ and $\beta 2$.¹³ The enzyme is active when a transient $\alpha 2\beta 2$ complex is formed.¹⁴ $\alpha 2$ contains the active site for nucleotide reduction and two allosteric effector binding sites that regulate the specificity and the rate of reduction.^{15–19} $\beta 2$ harbors the essential di-iron tyrosyl radical cofactor ($\text{Fe}_2^{\text{III}}\text{-Y}_{122}^{\cdot}$).^{20,21} During each turnover, $\text{Y}_{122}^{\cdot}\text{-}\beta 2$ oxidizes $\text{C}_{439}\text{-}\alpha 2$ to a thiyl radical, which subsequently initiates dNDP production.¹¹ There are X-ray structures of the individual subunits, and a docking model of the $\alpha 2\beta 2$ complex places $\text{Y}_{122}^{\cdot}$ at a distance of about 35 Å from C_{439} .^{22,23} 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 ($\text{Y}_{122} \leftrightarrow [\text{W}_{48}^?] \leftrightarrow \text{Y}_{356}$ in $\beta 2 \leftrightarrow \text{Y}_{731} \leftrightarrow \text{Y}_{730} \leftrightarrow \text{C}_{439}$ in $\alpha 2$). 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.^{4,14,24} Nevertheless, in wild-type (wt) *E. coli* RNR, the rate limiting step, conformational change(s) upon substrate and allosteric effector binding to $\alpha 2$, has prevented spectroscopic detection of any intermediates in this process.²⁹ The recent development of methods to site-specifically incorporate tyrosine analogs with altered pK_a s and reduction potentials has permitted the detection of pathway radical intermediates^{30–32} and, combined with state-of-the-art EPR spectroscopy,^{12,26,33,34} 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 $\beta 2$ and collinear PCET steps within the $\alpha 2$ subunit.^{12,33} However, the mechanism of the PCET process at the subunit interface between Y_{356} in $\beta 2$ and Y_{731} in $\alpha 2$ remains elusive, as structural information on the C-terminal 35 amino acids of $\beta 2$, including a putative proton acceptor E_{350} and Y_{356} (Fig. 1), is missing.²²

Our recent high-field (HF) EPR/ENDOR and DFT investigations using the 3-aminotyrosine mutants $\text{NH}_2\text{Y}_{730}\text{-}\alpha 2$ and

$\text{NH}_2\text{Y}_{731}\text{-}\alpha 2$, which generate the corresponding $\text{NH}_2\text{Y}^{\cdot}$ upon incubation with $\beta 2$, CDP (substrate) and ATP (allosteric effector), established that an unusual stacked conformation of residues 730 and 731, observed in some X-ray structures of $\alpha 2$ (ref. 23 and 36) (see ESI, Fig. S1†), occurs in the $\alpha 2\beta 2$ complex.^{12,33} However, the X-ray structure of $\text{NH}_2\text{Y}_{730}\text{-}\alpha 2$ (PDB 2XO4) alone exhibited multiple conformations for $\text{Y}_{731}\text{-}\alpha 2$, with one rotated away from $\text{NH}_2\text{Y}_{730}\text{-}\alpha 2$ toward the $\alpha 2\beta 2$ subunit interface.³⁰ This “flipped” conformation was accompanied by reorientations of R_{411} and N_{733} in $\alpha 2$. Further comparison of $\text{NH}_2\text{Y}_{730}\text{-}\alpha 2$, $\text{NH}_2\text{Y}_{731}\text{-}\alpha 2$ and $\text{NH}_2\text{Y}_{356}\text{-}\beta 2$ by HF EPR indicated that the electrostatic environment of all three transient $\text{NH}_2\text{Y}^{\cdot}$ s is strongly perturbed and that their hydrogen bond interactions are intrinsically different.^{12,33} Interestingly, one of our DFT models of the protein environment for $\text{NH}_2\text{Y}_{731}\text{-}\alpha 2$ required $\text{R}_{411}\text{-}\alpha 2$ to explain the perturbed g_x value observed and suggested that $\text{R}_{411}\text{-}\alpha 2$ approaches to $\text{NH}_2\text{Y}_{731}\text{-}\alpha 2$ within 2.6 Å (Fig. S1†).¹² Therefore, to examine the role of $\text{R}_{411}\text{-}\alpha 2$ during the PCET process in *E. coli* RNR, we generated two mutants: $\text{R}_{411}\text{A-}\alpha 2$ and the double mutant $\text{NH}_2\text{Y}_{731}/\text{R}_{411}\text{A-}\alpha 2$. Here, we report the incubation of $\text{NH}_2\text{Y}_{731}/\text{R}_{411}\text{A-}\alpha 2$ with $\beta 2/\text{CDP}$ and ATP, which generates the $\text{NH}_2\text{Y}_{731}^{\cdot}/\text{R}_{411}\text{A-}\alpha 2\beta 2$ 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 $\text{NH}_2\text{Y}_{731}^{\cdot}/\text{R}_{411}$ that is “flipped” towards the subunit interface in the $\alpha 2\beta 2$ complex. This is the first time an alternative conformation of any pathway tyrosine ($\text{NH}_2\text{Y}_{731}^{\cdot}$) 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, L-arabinose (ara), β -mercaptoethanol (β -ME), streptomycin sulfate and NH_2Y 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 $\text{R}_{411}\text{A-}\alpha 2$ and $\text{NH}_2\text{Y}_{731}/\text{R}_{411}\text{A-}\alpha 2$

The Quikchange kit (Stratagene) was used to generate each mutant according to the manufacturer's protocol. The templates pET28a-nrdA and pET28a-nrdA $\text{Y}_{731}\text{Z}^{30}$ were amplified with primer 5'-G CAG GAA CGT GCG TCT ACC GGT GCG ATC TAT ATT CAG AAC GTT GAC-3' and its reverse complement

Fig. 1 Current model of the long-range PCET in *E. coli* RNR. R_{411} is shown along with the redox active amino acid residues that are involved in this process ($\text{Y}_{122} \leftrightarrow [\text{W}_{48}^?] \leftrightarrow \text{Y}_{356}$ in $\beta 2 \leftrightarrow \text{Y}_{731} \leftrightarrow \text{Y}_{730} \leftrightarrow \text{C}_{439}$ in $\alpha 2$). W_{48} , D_{237} and E_{350} are shown in grey, because currently there is no evidence for their role in this process.¹⁴ The locations of Y_{356} and E_{350} are unknown, as they are within the flexible C-terminal tail (35 amino acids) of $\beta 2$. Red, blue and purple arrows represent electron transfer, proton transfer and collinear PCET pathways, respectively.

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.³⁰

Expression, purification and activity assays of R₄₁₁A- α 2 and NH₂Y₇₃₁/R₄₁₁A- α 2

(His)₆-wt- α 2 (2750 nmol min⁻¹ mg⁻¹) and wt- β 2 (7000 nmol min⁻¹ mg⁻¹), and 1.2 Y β 2 were expressed and purified by standard protocols.^{30,37,38} All α 2 mutants were pre-reduced with 30 mM DTT and 15 mM HU before use.²⁹ *E. coli* thioredoxin (TR, 40 U mg⁻¹) and thioredoxin reductase (TRR, 1800 U mg⁻¹) used in assays were isolated as previously described.^{39,40} (His)₆-NH₂Y₇₃₁- α 2 was purified as previously described.³⁰ Expression and purification of R₄₁₁A- α 2 and NH₂Y₇₃₁/R₄₁₁A- α 2 followed previous protocols,³⁰ except that the purification buffer (50 mM Tris, 5% glycerol, 1 mM PMSF, pH 7.6) for NH₂Y₇₃₁/R₄₁₁A- α 2 contained 1 mM TCEP. The yields of purified R₄₁₁A- α 2 and NH₂Y₇₃₁/R₄₁₁A- α 2 were 10–12 mg g⁻¹ and 6–7 mg g⁻¹ cell paste, respectively. The activity of R₄₁₁A- α 2 (0.2 μ M) and NH₂Y₇₃₁/R₄₁₁A- α 2 (1 μ M) was determined in the presence of 50-fold excess of wt- β 2 with 3 mM ATP, 1 mM [³H]-CDP (4850 cpm nmol⁻¹), 30 μ M TR, 0.5 μ M TRR, and 1 mM NADPH in assay buffer (50 mM HEPES, 1 mM EDTA, 15 mM MgSO₄, pH 7.6). The amount of dCDP was determined by the method of Steeper and Steuart.⁴¹ For single turnover experiments, NH₂Y₇₃₁/R₄₁₁A- α 2 (5 μ M) was incubated with wt- β 2 (5 μ M), 3 mM ATP, and 1 mM [³H]-CDP (20 000 cpm nmol⁻¹) in assay buffer. The dissociation constant (*K_d*) for R₄₁₁A- α 2 and wt- β 2 was determined in H₂O and D₂O buffers by the competitive inhibition assay⁴² (SI-2, Fig. S2†).

Samples for HF EPR and PELDOR spectroscopy

NH₂Y₇₃₁/R₄₁₁A- α 2 and wt- β 2 were mixed 1:1 to a final concentration of 160–180 μ M in D₂O assay buffer as previously described.^{32,34} These protein concentrations resulted in >95% binding between subunits. The reaction was initiated at room temperature by adding CDP and ATP to final concentrations of 1 and 3 mM, respectively. The reactions were manually freeze-quenched 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 NH₂Y₇₃₁ accounted for 30–33% of the total spin for all the samples used in this work, which was similar to the yields reported previously.^{30,32}

HF pulsed EPR spectroscopy

Echo-detected (ESE: $\pi/2 - \tau - \pi - \text{echo}$) EPR spectra at 263 GHz were recorded on a Bruker Elexsys E780 quasi optical spectrometer using a single mode (TE₀₁₁) cylindrical resonator (E9501610 – 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 CV2033S) 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 μ L in 0.84 mm OD capillaries (Wilma 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 μ L in 1.6 mm OD capillaries (Wilma 222T-RB).

Processing and simulation of EPR spectra

Spectra were processed by phasing and 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_R2014b.⁴³ EPR spectra were simulated using the EasySpin-4.5.5 “pepper”-routine which was run in MATLAB.⁴⁴

DFT calculations

DFT calculations were performed with the ORCA 3.0.0 program package.⁴⁵ The geometry optimization of the neutral NH₂Y[•] was performed using the unrestricted B3LYP^{46–48} hybrid density functional in combination with the def2-TZVPP basis set and def2-TZVPP/JK auxiliary basis set.^{49,50} To take into account the electrostatic environment of the radical intermediate at the protein interface, a solvation model (COSMO⁵¹) with the polarity of ethanol ($\epsilon = 24$) was used. Otherwise, Grimme's dispersion correction^{52,53} and RIJCOSX⁵⁴ approximations were employed. The energy converged to 10⁻⁹ E_h. The hyperfine couplings and *g* values were calculated using NH₂Y[•]-C₄ as the gauge origin.^{55,56} The def2-TZVPP basis set was consistent with the geometry optimization step.⁵⁰ The C2–C1–C β –C α 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 ESI.†

PyMOL models

The docking model refers to the α 2 β 2 complex structure generated from the individual wt- α 2 and wt- β 2 X-ray structures.^{22,23} In order to predict distances, the mutant *E. coli* RNR structure (PDB 2XO4)³⁰ was overlaid with the wt- α 2 structure in the docking model²³ 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- α 2, NH₂Y₇₃₁/R₄₁₁A- α 2 and ND₂Y₇₃₁/R₄₁₁A- α

Our recent studies on NH₂Y₇₃₁- α 2 (ref. 12) suggested that R₄₁₁ might interact with NH₂Y₇₃₁, partially accounting for the measured EPR and ENDOR parameters. To investigate this proposal, R₄₁₁A- α 2 was generated and characterized. Because the mutation is proposed to be at the interface of α 2 and β 2, the dissociation constant (K_d) for subunit interactions was also examined and was determined to be $0.94 \pm 0.33 \mu\text{M}$ (Fig. S2A†), ~ 5 fold higher than that for wt- α 2 ($0.18 \mu\text{M}$).⁴² Under these conditions, this mutant was shown to have a specific activity of $467 \pm 22 \text{ nmol min}^{-1} \text{ mg}^{-1}$, 17% of that of the wt enzyme ($2750 \text{ nmol min}^{-1} \text{ mg}^{-1}$). The reduced activity and weaker subunit binding suggest that R₄₁₁ plays a functional role.

Furthermore, we characterized the role of R₄₁₁ in the oxidation of Y₇₃₁- α 2 by generating the double mutant NH₂Y₇₃₁/R₄₁₁A- α 2. The K_d for subunit interactions between NH₂Y₇₃₁/R₄₁₁A- α 2 and wt- β 2 was determined to be $8 \pm 1 \text{ nM}$ (Fig. S2C†), which is consistent with the formation of a tight complex when a NH₂Y[•] is generated.²⁸ Its specific activity was $13 \pm 3 \text{ nmol min}^{-1} \text{ mg}^{-1}$, 0.4% of the specific activity of wt-RNR and in the range of contaminating wt- α 2 activity.³² A more sensitive, one turnover assay was then employed to determine if this double mutant could generate any dCDP. When pre-reduced NH₂Y₇₃₁/R₄₁₁A- α 2 was mixed with wt- β 2, CDP, and ATP for 5 min, only $0.036 \pm 0.018 \text{ dCDP}/\alpha$ 2 was observed, consistent with contaminating wt- α 2. Thus, the double mutant is unable to make detectable dCDP, which is not unexpected, given the specific activities of the R₄₁₁A and the NH₂Y₇₃₁- α 2 mutants (see also SI-3 and Fig. S3†).

We next investigated whether NH₂Y₇₃₁[•] could be generated by NH₂Y₇₃₁/R₄₁₁A- α 2, despite its inability to make dCDPs. NH₂Y₇₃₁/R₄₁₁A- α 2, wt- β 2, CDP and ATP were studied by stopped-flow (SF) spectroscopy and the reaction was monitored at 320 nm, the absorption feature associated with the NH₂Y[•] (Fig. S4,† red). The data were split into two time domains: 5 ms to 6 s and 25 s to 100 s. In the first time domain, NH₂Y₇₃₁[•] formation was fit to a double exponential with k_{fast} of $3.6 \pm 0.5 \text{ s}^{-1}$ (amplitude 8%) and k_{slow} of $0.47 \pm 0.03 \text{ s}^{-1}$ (amplitude 21%) (Table S1†). The rate constants for NH₂Y₇₃₁[•] in the single mutant control were similar: k_{fast} of $9.6 \pm 0.6 \text{ s}^{-1}$ and k_{slow} of $0.8 \pm 0.1 \text{ s}^{-1}$. However, in this case, the fast phase accounted for 27% and the slow phase accounted for 13% of the NH₂Y₇₃₁[•]. The biphasic kinetics of NH₂Y₇₃₁[•] formation in both cases is attributed to multiple conformations that give rise to NH₂Y₇₃₁[•].³² From 25 s to 100 s, NH₂Y₇₃₁[•] in the double mutant reaction disappeared with a k_{obs} of $0.02 \pm 0.003 \text{ s}^{-1}$, while with the single mutant, disappearance occurred with a k_{obs} of $0.005 \pm 0.002 \text{ s}^{-1}$. 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- α 2, the 9 GHz EPR spectrum of the sample generated from the reaction of NH₂Y₇₃₁/R₄₁₁A- α 2 with wt- β 2, ATP, and

CDP quenched after 25 s was recorded and is shown in Fig. S5A and C.† Subsequent to subtraction of Y₁₂₂[•], 32% of the total spin is associated with NH₂Y₇₃₁[•]/R₄₁₁A- α 2 with no spin loss. This result is similar to that of the single mutant, NH₂Y₇₃₁[•].^{30,32} 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- α 2

To examine the generated ND₂Y₇₃₁[•]/R₄₁₁A- α 2, we took advantage of the proximity of Y₁₂₂[•] to the di-iron cluster and its altered relaxation properties. Pulsed EPR spectra of ND₂Y₇₃₁[•]/R₄₁₁A- α 2 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- α 2 EPR spectrum at 34 GHz is mainly dominated by the large hf couplings with the deuterons of the amino group and the two C β -methylene protons.³⁴ 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- α 2 are best resolved at 263 GHz and are consistent with the values from our previous ND₂Y[•] studies.^{12,33} The 94 GHz spectra reveal differences in the hf splitting of the C β -methylene protons (Fig. 2A, marked with an arrow): the large hf splitting of the C β -methylene proton visible in the central line of ND₂Y₇₃₁[•]- α 2 (red) is missing in ND₂Y₇₃₁[•]/R₄₁₁A- α 2 (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 reported³⁴ hf coupling for ¹⁴N is used, are consistent with the NH₂Y₇₃₁[•] generated in the NH₂Y₇₃₁[•]/R₄₁₁A- α 2/ β 2 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[•]/Y₇₃₀F- α 2 and NH₂Y₇₃₀[•]/C₄₃₉A- α 2, 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- α and ND₂Y₇₃₁[•]- α 2. This is unexpected because the *g_x* value is affected by the electrostatic environment of a radical,⁵⁷ and the R₄₁₁A mutation has changed the local environment of ND₂Y₇₃₁[•], as demonstrated by the substantial changes in the C β -methylene ¹H couplings (Table 1). These couplings are related to the dihedral angle $\theta_{\text{C}\beta}$ between the C β -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.³⁴ The dihedral angle can be extracted from the McConnell equation ($a_{\text{iso(C}\beta\text{-H)}} = B_1 \times \rho_{\text{C1}} \times \cos^2 \theta_{\text{C}\beta}$),⁵⁸ which provides a semi-empirical relationship for the observed isotropic constant *a_{iso}*. The C2-C1-C β -C α angle of

Fig. 2 (A) Derivative EPR spectra (solid black lines) at 34 GHz (top), 94 GHz (middle) and 263 GHz (bottom) of ND₂Y₇₃₁*/R₄₁₁A-α2 with the corresponding simulations (dashed black lines). The 94 GHz EPR spectrum of ND₂Y₇₃₁*/α2 in the single mutant (solid red line) with the corresponding simulation (dashed red line) is shown for comparison. The difference between the two spectra around g_y is marked with an arrow. The glass signal is marked with an asterisk. Exp. conditions (34 GHz): $\pi/2 = 6$ ns, $\tau = 280$ ns, shot repetition time = 6 ms, shots/point = 80, number of scans = 10; (94 GHz): $\pi/2 = 30$ ns, $\tau = 280$ ns, shot repetition time = 5 ms, shots/point = 100, number of scans = 50–100; (263 GHz): $\pi/2 = 40$ ns, $\tau = 270$ ns, shot repetition time = 6 ms, shots/point = 250, number of scans = 36. Structure of NH₂Y* is shown in the inset. (B) Orientation of the Cβ-methylene protons with respect to the phenol ring, as extracted from the observed hyperfine couplings. (C) a_{iso} as a function of the dihedral angle for each Cβ-methylene proton, calculated from a DFT model for NH₂Y* (Fig. S6†).

Table 1 Summary of g values and large hf couplings (>8 MHz) of ND₂Y₇₃₁* in the double and single mutants^a

Sample	g values			a_{iso} (MHz)		
	g_x	g_y	g_z	Hβ ₁	Hβ ₂	¹⁴ N
ND ₂ Y ₇₃₁ */R ₄₁₁ A-α	2.0051	2.0041	2.0022	10	10	12
ND ₂ Y ₇₃₁ */α ^b	2.0051	2.0041	2.0022	22	9	12

^a Uncertainties in the g values and hf couplings are about 0.00005 and up to 10%, respectively, as obtained from the spectral simulations. ^b g values and hf couplings were reported in ref. 12 and 34, respectively.

ND₂Y₇₃₁*/R₄₁₁A-α2 is estimated to be $\approx 90^\circ$ by using B_1 of 162 MHz (ref. 59) for tyrosyl radicals, an electron spin density ρ_{C1} of 0.214,¹² and an isotropic Cβ-methylene proton hf coupling $a_{\text{iso}} = 10 \pm 1$ MHz (Table 1). This dihedral angle is indeed consistent with the hf couplings of the two Cβ-methylene ¹H 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 NH₂Y*, 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 C₁ (Fig. 2B). In this calculation, a $\theta_{C\beta}$ angle of 90° corresponds to $a_{\text{iso}} = 9 \pm 3$ MHz (grey area in Fig. 2C) for both Cβ-methylene protons, Hβ_{2/1}.

ENDOR for detection of hydrogen bonds to ND₂Y₇₃₁*/R₄₁₁A-α2

Given that the R₄₁₁A mutation had little effect on g_x , ²H ENDOR spectroscopy was used to further examine a possible correlation of the observed g_x value ($g_x = 2.0051$) with the hydrogen bonding environment. Fig. 3 illustrates the ²H Mims ENDOR spectra of ND₂Y₇₃₁*/α2 and ND₂Y₇₃₁*/R₄₁₁A-α2. Both spectra contain a broad signal that extends over ± 2 MHz, arising from the strongly coupled amino deuterons, which is a common feature of ND₂Y* Mims ENDOR spectra.^{12,33} However, we observe that the ²H hf tensor previously assigned to the moderately strong hydrogen bond between Y₇₃₀ and Y₇₃₁ in ND₂Y₇₃₁*/α2, which is almost perpendicular to the tyrosine ring plane,¹² is absent in the ND₂Y₇₃₁*/R₄₁₁A-α2 spectrum. Therefore, the hydrogen bonding environment of NH₂Y₇₃₁*/R₄₁₁A-α2 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 ND₂Y₇₃₁*/R₄₁₁A-α2 can be excited at 34 GHz by using very short microwave pulses,

Fig. 3 34 GHz ^2H ENDOR spectra of $\text{ND}_2\text{Y}_{731}^*/\text{R}_{411}\text{A}-\alpha 2$ (solid black line) and $\text{ND}_2\text{Y}_{731}^*-\alpha 2$ (solid red line). Simulations are shown with dashed lines. The hydrogen bond peaks, "H-bond", assigned to a deuteron located between $\text{Y}_{730}-\alpha 2$ and $\text{ND}_2\text{Y}_{731}^*-\alpha 2$ (ref. 12), are not observed for $\text{ND}_2\text{Y}_{731}^*/\text{R}_{411}\text{A}-\alpha 2$. The inset shows the structured and broad matrix line of $\text{ND}_2\text{Y}_{731}^*/\text{R}_{411}\text{A}-\alpha 2$. Exp. conditions: Mims ENDOR with $\pi/2 = 6$ ns, $\tau = 200$ ns and 320 ns for the inset, shot repetition time = 15 ms, random RF acquisition⁶⁰ with 1 shot/point, acquisition time = 15–20 h, $T = 70$ K. Excitation in the EPR line is set to $B_0||g_y$. ENDOR spectra are centered at the Larmor frequency of ^2H , $\nu_0 = 7.9$ MHz at 1.2 T.

and thus hf couplings cannot be missed due to orientation selective effects.

Although no exchangeable moderately strong hydrogen bonds ($r_{\text{O-H}} \sim 1.7\text{--}2$ Å) to $\text{ND}_2\text{Y}_{731}^*/\text{R}_{411}\text{A}-\alpha 2$ are observed, the ENDOR spectrum of $\text{ND}_2\text{Y}_{731}^*/\text{R}_{411}\text{A}-\alpha 2$ exhibits a broad and structured matrix line, which is associated with weak hf interactions of the radical with distant nuclei⁶¹ (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 $\text{ND}_2\text{Y}_{731}^*/\text{R}_{411}\text{A}-\alpha 2$ is reminiscent of the one previously observed for $\text{ND}_2\text{Y}_{356}^*-\beta 2$, also located at the subunit interface and likely surrounded by a defined hydrogen bonded network of water molecules.¹² The similarity between the ENDOR spectra of $\text{ND}_2\text{Y}_{356}^*-\beta 2$ and $\text{ND}_2\text{Y}_{731}^*/\text{R}_{411}\text{A}-\alpha 2$ suggests a similar origin for the g_x values in these two mutants, which is distinct from that in $\text{ND}_2\text{Y}_{731}^*-\alpha 2$. As noted above, in the case of $\text{ND}_2\text{Y}_{356}^*-\beta 2$ the g_x value was also strongly shifted ($\text{NH}_2\text{Y}_{356}^*$: $g_x = 2.0049$ vs. free NH_2Y^* : $g_x = 2.0061$ (ref. 33)). Therefore, we propose that the g_x -shift in $\text{NH}_2\text{Y}_{731}^*/\text{R}_{411}\text{A}-\alpha 2$, as well as in $\text{ND}_2\text{Y}_{356}^*-\beta 2$, 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 $\text{NH}_2\text{Y}_{731}^*-\alpha 2$ resulted in similar g -shifts.¹² Overall, these data clearly indicate that the molecular orientation of $\text{ND}_2\text{Y}_{731}^*/\text{R}_{411}\text{A}-\alpha 2$ is different to that of $\text{ND}_2\text{Y}_{731}^*-\alpha 2$ and is affected by $\text{R}_{411}\text{A}-\alpha 2$ substitution.

PELDOR gives evidence for a conformational change in $\text{ND}_2\text{Y}_{731}^*/\text{R}_{411}\text{A}-\alpha 2$

Our previous PELDOR studies²⁶ have demonstrated that half sites reactivity of *E. coli* RNR allows for the detection of the diagonal inter-spin distance between Y_{122}^* in one $\alpha\beta$ pair and any radical trapped in the second $\alpha\beta$ pair (Fig. 4A).^{25,62} To gain insight into the location of $\text{NH}_2\text{Y}_{731}^*/\text{R}_{411}\text{A}-\alpha 2$, 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 line^{63–66} (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 $\Delta r = \pm 2.7$ Å. We note that the error in the peak distance is much less than the distribution and is estimated to be $\leq \pm 0.5$ Å. The width of the distance distribution is slightly larger than in previous measurements within the *E. coli* RNR $\alpha 2\beta 2$ complex,^{25,26,62} suggesting more conformational heterogeneity for $\text{ND}_2\text{Y}_{731}^*/\text{R}_{411}\text{A}-\alpha 2$, consistent with the observed flexibility of this residue. Nevertheless, the results clearly indicate that the R_{411} mutation induces a conformational change of $\text{ND}_2\text{Y}_{731}^*$ 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 $\text{ND}_2\text{Y}_{731}^*-\alpha 2$.²⁶ This distance might appear to be rather close to the initial distance (prior to turnover) between the two stable Y_{122}^* s, that is 33.1 ± 0.2 Å.⁶² To confirm our assignment, we recorded PELDOR experiments at higher temperature (50 K), in which the $\text{Y}_{122}^*-\beta 2$ contribution to the re-focused echo is filtered and $\text{ND}_2\text{Y}_{731}^*-\alpha 2$ is the only radical species detected (Fig. S9†). However, $\text{Y}_{122}^*-\beta 2$ 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 $\text{Y}_{122}^*-\text{ND}_2\text{Y}_{731}^*$ and cannot be associated with the $\text{Y}_{122}^*-\text{Y}_{122}^*$ 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 $\Delta r = \pm 2.0$ Å, and thus validated our assignment (see Fig. S9†).

To gain more insight into the conformation of $\text{NH}_2\text{Y}_{731}^*/\text{R}_{411}\text{A}-\alpha 2$ and the role of R_{411} , we examined the available X-ray structures of *E. coli* $\alpha 2$ s in the R_{411} region. In the structure of *E. coli* $\text{NH}_2\text{Y}_{730}-\alpha 2$ (2XO4),³⁰ Y_{731} is flipped away from $\text{NH}_2\text{Y}_{730}$, as shown in Fig. 5. This altered conformation is compared with a second α in the unit cell, in which the Y_{731} is not flipped. To match the 35 Å distance observed by PELDOR spectroscopy, the aromatic ring of $\text{NH}_2\text{Y}_{731}$ must rotate away from Y_{730} toward the $\beta 2$ subunit, as observed for Y_{731} in the *E. coli* $\text{Y}_{730}\text{NH}_2\text{Y}-\alpha 2$ structure (Fig. 5).

Fig. 4 (A) Diagonal distance between Y_{122}^* in $\beta 2$ and $ND_2Y_{731}^*/R_{411}A-\alpha 2$. (B) The ESE spectrum of $ND_2Y_{731}^*/R_{411}A-\alpha 2$ is composed of the $Y_{122}^*-\beta 2$ spectrum (dashed and dotted black line) and the $ND_2Y_{731}^*-\alpha 2$ spectrum (dotted black line). Detect (D) and pump (P) frequency positions for each PELDOR measurement are displayed by red and black arrows, respectively. Exp. conditions (EPR): $\pi/2 = 6$ ns, $\tau = 280$ ns, shot repetition time = 120 ms, shots/point = 10, 4 scans, $T = 20$ K. (C) Background- and phase-corrected, normalized 34 GHz PELDOR time traces of three experimental setups (D_1 , D_2 , D_3). The sum of the three traces ($D_1 + D_2 + D_3$) was analyzed by DeerAnalysis 2015 (ref. 67) and is shown in black with the fitting overlaid in a solid red line. Detect/pump π pulse lengths for D_1 , D_2 and D_3 were 30 ns/12 ns, 30 ns/12 ns and 20 ns/14 ns, respectively. The frequency separation between detect and pump pulses was 80 MHz for all data sets. (D) Distance distribution obtained from the analysis in (C). Asterisks indicate artifacts attributed to the analysis procedure.

Fig. 5 The *E. coli* $Y_{730}NH_2Y-\alpha 2$ structure ($2XO4$)³⁰ in green shows the reoriented Y_{731} overlaid with the stacked Y_{731} in a different monomer (blue) of the unit cell. The diagonal distances between the “flipped” and non-flipped Y_{731} and Y_{122} are 3.5 nm and 3.9 nm, respectively. These distances, which are between two phenolic oxygen atoms of the tyrosine residues, are based on the alignment with the *E. coli* $\alpha 2\beta 2$ docking model. Residue Y_{356} is shown in grey with a “?” because its position is unknown.

This reorientation is also supported by the ENDOR data, which indicate that the stacked conformation between $NH_2Y_{731}^*$ and Y_{730} with a shared, perpendicular hydrogen bond

is absent in $NH_2Y_{731}^*/R_{411}A-\alpha 2$, and that the radical is instead surrounded by weakly coupled hydrogen bonds, likely water molecules at the $\alpha 2\beta 2$ subunit interface. The exposure of $NH_2Y_{731}^*/R_{411}A-\alpha 2$ 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 $NH_2Y_{731}-\alpha 2$ 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_2Y_{731} and Y_{122} exceeds the observed distance by ≥ 2 Å. We note that the “flipped” conformation has not been observed in the single mutant $NH_2Y_{731}-\alpha 2$ or in the double mutant $NH_2Y_{731}/Y_{730}F-\alpha 2$, in which Y_{731} lacks its hydrogen bonding partner,¹² 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 $\beta 2$.^{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- $\alpha 2$ unmasks a new conformation of pathway residue 731 in the $\alpha 2\beta 2$ 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 $\alpha 2$, as well as through the $\alpha 2\beta 2$ interface. First, R₄₁₁ appears to play a role in the stabilization of the stacked conformation of Y₇₃₁ and Y₇₃₀, and thus in the facilitation of collinear PCET within the $\alpha 2$ 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 $\beta 2$. 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.^{6,7} This opens up a new hypothesis for the PCET mechanism between residues Y₇₃₁- $\alpha 2$ and Y₃₅₆- $\beta 2$, which could involve a gated conformational change in Y₇₃₁- $\alpha 2$ 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 Y₇₃₁- $\alpha 2$ and Y₃₅₆- $\beta 2$ in PCET through the subunit surface.

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