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

Müge Kasanmascheff,$^{†,ab}$ Wanyyu Lee,$^{†,c}$ Thomas U. Nick,$^a$ JoAnne Stubbe$^{*c}$ and Marina Bennati*ab

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 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 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 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).

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. 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. 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.

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. 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. In this paper, we use the *Escherichia coli* (*E. coli*) class I 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 tyrosine residues, one on each subunit, and a water interface between the subunits.

---

*Max Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany. E-mail: marina.bennati@mpibpc.mpg.de

†Department of Chemistry, University of Göttingen, 37077 Göttingen, Germany

‡Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA. E-mail: stubbe@mit.edu

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c5sc03460d

†† These authors contributed equally.
The *E. coli* RNR consists of two homodimeric subunits, α2 and β2.1,2 The enzyme is active when a transient αβ2 complex is formed.1,2 α2 contains the active site for nucleotide reduction and two allosteric effector binding sites that regulate the specificity and the rate of reduction.3-11 β2 harbors the essential di-iron tyrosyl radical cofactor (FeIII-Y122)/FeII-Y122.12,24 During each turnover, Y122/β2 oxidizes C439Yα2 to a thyl radical, which subsequently initiates dNTP formation.1 There are X-ray structures of the individual subunits, and a docking model of the αβ2 complex places Y122 at a distance of about 35 Å from C439.12,23 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 → [W667] → Y356 in β2 to Y731 → Y730 → C439 in α2). Biochemical14 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.14,29 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.29 The recent development of methods to site-specifically incorporate tyrosine analogs with altered pH sensitivity and reduction potentials has permitted the detection of pathway radical intermediates30-32 and, combined with state-of-the-art EPR spectroscopy,12,26,31,33 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.15,16 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.16

Our recent high-field (HF) EPR/ENDOR and DFT investigations using the 3-aminotyrosine mutants NH2Y356/α2 and NH2Y731/β2, 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 αβ2 complex.12,26 However, the X-ray structure of NH2Y370/α2 (PDB 2XO4) alone exhibited multiple conformations for Y371/α2, with one rotated away from NH2Y370/α2 toward the α2β2 subunit interface.36 This “flipped” conformation was accompanied by reorientations of R411 and N733 in α2. Further comparison of NH2Y370/α2, NH2Y731/β2 and NH2Y356/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/β2 required R411α2 to explain the perturbed gα value observed and suggested that R411α2 approaches to NH2Y731/α2 within 2.6 Å (Fig. S1†).12 Therefore, to examine the role of R411α2 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) spectroscopy, we have provided evidence for a new conformation of NH2Y371/β2 that is “flipped” towards the subunit interface in the αβ2 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), 2XTY media, M9 Minimal Salts, L-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 pET28α-nrdA and pET28α-nrdA Y731Z 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.
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.²⁸

Expression, purification and activity assays of R₄₁₁A-α₂ and NH₂₃₁/R₄₁₁A-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.³⁶,³⁷ All α₂ 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.²⁹,⁴⁰ (His)₆-NH₂₃₁-α₂ was purified as previously described.³⁴ Expression and purification of R₄₁₁A-α₂ and NH₂₃₁/R₄₁₁A-α₂ followed previous protocols,³⁶ except that the purification buffer (50 mM Tris, 5% glycerol, 1 mM PMSF, pH 7.6) for NH₂₃₁/R₄₁₁A-α₂ contained 1 mM TCEP. The yields of purified R₄₁₁A-α₂ and NH₂₃₁/R₄₁₁A-α₂ were 10–12 mg g⁻¹ and 6–7 mg g⁻¹ cell paste, respectively. The activity of R₄₁₁A-α₂ (0.2 μM) and NH₂₃₁/R₄₁₁A-α₂ (1 μM) was determined in the presence of 50-fold excess of wt-β₂ with 3 mM ATP, 1 mM [¹H]-CDP (4850 cpm nmol⁻¹), 30 μM TR, 0.5 μM 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.³¹ For single turnover experiments, NH₂₃₁/R₄₁₁A-α₂ (5 μM) was incubated with wt-β₂ (5 μM), 3 mM ATP, and 1 mM [¹H]-CDP (20 000 cpm nmol⁻¹) in assay buffer. The dissociation constant (K_d) for R₄₁₁A-α₂ and wt-β₂ was determined in H₂O and D₂O buffers by the competitive inhibition assay⁴⁵ (SI-2, Fig. S2†).

Samples for HF EPR and PELDOR spectroscopy

NH₂₃₁/R₄₁₁A-α₂ and wt-β₂ were mixed 1:1 to a final concentration of 160–180 μM in D₂O assay buffer as previously described.²⁴,¹² 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 (OD₃) 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 NH₂₃₁-α₂ accounted for 30–33% of the total spin for all the samples used in this work, which was similar to the yields reported previously.²⁹,³²

HF pulsed EPR spectroscopy

Echo-detected (ESE: π/2 – τ – π – echo) EPR spectra at 263 GHz were recorded on a Bruker Elexys 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 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 TW 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 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.⁴⁶ The geometry optimization of the neutral NH₂₃₁ in *E. coli* RNR was performed using the unrestricted B3LYP⁵⁰–⁵⁴ hybrid density functional in combination with the def2-TZVPP basis set and def2-TZVPP/JK auxiliary basis set.⁵⁹ To take into account the electrostatic environment of the radical intermediate at the protein interface, a solvation model (COSMO) was used. Otherwise, Grimme’s dispersion correction²⁵–²⁹ and RIJCOSX approximations were employed. The energy converged to 10⁻⁹ Eh. The hyperfine couplings and g values were calculated using NH₂₃₁-Y⁻C₄ as the gauge origin.²⁵,²⁶ The def2-TZVPP basis set was consistent with the geometry optimization step.²⁹ The C₂–C₁–C₆–C₇ dihedral angle of the NH₂₃₁ 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.⁵⁰ In order to predict distances, the mutant *E. coli* RNR structure (PDB 2X04) 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 R_{411}A-a2, NH$_2$Y$_{731}$/R$_{411}$A-a2 and ND$_2$Y$_{731}$/R$_{411}$A-a2

Our recent studies on NH$_2$Y$_{731}$-a2 (ref. 12) suggested that R$_{411}$ might interact with NH$_2$Y$_{731}$, partially accounting for the measured EPR and ENDOR parameters. To investigate this proposal, R$_{411}$A-a2 was generated and characterized. Because the mutation is proposed to be at the interface of a2 and b2, the dissociation constant ($K_d$) for subunit interactions was also examined and was determined to be 0.94 ± 0.33 μM (Fig. S2A†), ~5 fold higher than that for wt-a2 (0.18 μM). Under these conditions, this mutant was shown to have a specific activity of 467 ± 22 nmol min$^{-1}$ mg$^{-1}$, 17% of that of the wt enzyme (2750 nmol min$^{-1}$ mg$^{-1}$). The reduced activity and weaker subunit binding suggest that R$_{411}$ plays a functional role.

Furthermore, we characterized the role of R$_{411}$ in the oxidation of Y$_{731}$-a2 by generating the double mutant NH$_2$Y$_{731}$/R$_{411}$A-a2. The $K_d$ for subunit interactions between NH$_2$Y$_{731}$/R$_{411}$A-a2 and wt-b2 was determined to be 8 ± 1 nM (Fig. S2C†), which is consistent with the formation of a tight complex when a NH$_2$Y is generated.† Its specific activity was 13 ± 3 nmol min$^{-1}$ mg$^{-1}$, 0.4% of the specific activity of wt-RNR and in the range of contaminating wt-a2 activity. A more sensitive, one turnover assay was then employed to determine if this double mutant could generate any dCDP. When pre-reduced NH$_2$Y$_{731}$/R$_{411}$A-a2 was mixed with wt-b2, CDP, and ATP for 5 min, only 0.036 ± 0.018 dCDP/a2 was observed, consistent with contaminating wt-a2. Thus, the double mutant is unable to make detectable dCDP, which is not unexpected, given the specific activities of the R$_{411}$A and the NH$_2$Y$_{731}$-a2 mutants (see also SI-3 and Fig. S3†).

We next investigated whether NH$_2$Y$_{731}$-c1 could be generated by NH$_2$Y$_{731}$/R$_{411}$A-a2, despite its inability to make dCDPs. NH$_2$Y$_{731}$/R$_{411}$A-a2, wt-b2, CDP and ATP were studied by stopped-flow (SF) spectroscopy and the reaction was monitored at 320 nm, the absorption feature associated with the NH$_2$Y (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, NH$_2$Y$_{731}$-c1 formation was fit to a double exponential with $k_{fast}$ of 3.6 ± 0.5 s$^{-1}$ (amplitude 8%) and $k_{slow}$ of 0.47 ± 0.03 s$^{-1}$ (amplitude 21%) (Table S1†). The rate constants for NH$_2$Y$_{731}$-c1 in the single mutant control were similar: $k_{fast}$ of 9.6 ± 0.6 s$^{-1}$ and $k_{slow}$ of 0.8 ± 0.1 s$^{-1}$. However, in this case, the fast phase accounted for 27% and the slow phase accounted for 13% of the NH$_2$Y$_{731}$-c1. The biphasic kinetics of NH$_2$Y$_{731}$-c1 formation in both cases is attributed to multiple conformations that give rise to NH$_2$Y$_{731}$-c1. From 25 s to 100 s, NH$_2$Y$_{731}$-c1 in the double mutant reaction disappeared with a $k_{obs}$ of 0.02 ± 0.003 s$^{-1}$, while with the single mutant, disappearance occurred with a $k_{obs}$ of 0.005 ± 0.002 s$^{-1}$. Analysis of the Y$_{122}$-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 NH$_2$Y$_{731}$-a2, the 9 GHz EPR spectrum of the sample generated from the reaction of NH$_2$Y$_{731}$/R$_{411}$A-a2 with wt-b2, ATP, and CDP quenched after 25 s was recorded and is shown in Fig. S5A and C.† Subsequent to subtraction of Y$_{122}$-c1, 32% of the total spin is associated with NH$_2$Y$_{731}$/R$_{411}$A-a2 with no spin loss. This result is similar to that of the single mutant, NH$_2$Y$_{731}$-c1.† 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 R$_{411}$. Therefore, the role of R$_{411}$ in the RT pathway was further studied with advanced EPR spectroscopy.

HF EPR of ND$_2$Y$_{731}$/R$_{411}$A-a2

To examine the generated ND$_2$Y$_{731}$/R$_{411}$A-a2, we took advantage of the proximity of Y$_{122}$ to the di-iron cluster and its altered relaxation properties. Pulsed EPR spectra of ND$_2$Y$_{731}$/R$_{411}$A-a2 at 34, 94 and 263 GHz were recorded in D$_2$O buffer at 70 K and are shown in Fig. 2A. The use of D$_2$O considerably simplifies the EPR spectra due to the absence of $^1$H hyperfine (hf) splittings arising from the amino protons. The ND$_2$Y$_{731}$/R$_{411}$A-a2 EPR spectrum at 34 GHz is mainly dominated by the large hf couplings with the deuteron of the amino group and the two Cβ-methylene protons. 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 ND$_2$Y$_{731}$/R$_{411}$A-a2 are best resolved at 263 GHz and are consistent with the values from our previous ND$_2$Y studies.12,35 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 ND$_2$Y$_{731}$/R$_{411}$A-a2 (red) is missing in ND$_2$Y$_{731}$/R$_{411}$A-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 $^{14}$N is used, are consistent with the NH$_2$Y$_{731}$-c1 generated in the NH$_2$Y$_{731}$/R$_{411}$A-a2/b2 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, NH$_2$Y$_{731}$/Y$_{330}$F-a2 and NH$_2$Y$_{731}$/C$_{430}$P-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 ND$_2$Y$_{731}$/R$_{411}$A-a2 and ND$_2$Y$_{731}$/R$_{411}$A-a2. This is unexpected because the $g_z$ value is affected by the electrostatic environment of a radical,57 and the R$_{411}$A mutation has changed the local environment of ND$_2$Y$_{731}$-c1, as demonstrated by the substantial changes in the Cβ-methylene $^1$H 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 C$_1$ (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 ($\Delta_{iso(CB-H)} = B_1 \times P_{C1} \times \cos^2 \theta_{CB}$),44 which provides a semi-empirical relationship for the observed isotropic constant $\Delta_{iso}$. The C2–C1–Cβ–Cz angle of

This journal is © The Royal Society of Chemistry 2016
a function of the dihedral angle for each C correponding simulations (dashed black lines). The 94 GHz EPR spectrum of ND2Y731
The glass signal is marked with an asterisk. Exp. conditions (34 GHz): number of scans

resonances being indistinguishable, as reported in Table 1 and

Consistent with the hf couplings of the two C-

ND2Y731
ND2Y731
values and hf couplings were reported in ref. 12 and 34, respectively. uncertainties in the

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>(g_x)</th>
<th>(g_y)</th>
<th>(g_z)</th>
<th>(a_{iso}) (MHz)</th>
<th>(H_{\beta 1})</th>
<th>(H_{\beta 2})</th>
<th>(^{14})N</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND2Y(\text{Y731}^{+}/R_{411}\alpha) (\alpha)</td>
<td>2.0051</td>
<td>2.0041</td>
<td>2.0022</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>ND2Y(\text{Y731}^{+}/R_{411}\alpha) (\alpha)</td>
<td>2.0051</td>
<td>2.0041</td>
<td>2.0022</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>(^{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.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND2Y\(\text{Y731}^{+}/R_{411}\alpha\) \(\alpha\) is estimated to be \(\approx 90^\circ\) by using \(B_1\) of 162 MHz (ref. 59) for tyrosyl radicals, an electron spin density \(\rho_{C1}\) of 0.214, and an isotropic C\(\beta\)-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 C\(\beta\)-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\(\text{H2Y}^+\), 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\(^\circ\) corresponds to \(a_{iso} = 9 \pm 3\) MHz (grey area in Fig. 2C) for both C\(\beta\)-methylene protons, \(H_{\beta 1/2}\).

ENDOR for detection of hydrogen bonds to ND2Y\(\text{Y731}^{+}/R_{411}\alpha\) \(\alpha\)Given that the \(R_{411}\alpha\) mutation had little effect on \(g_s\), \(^3\)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 \(^3\)H Mims ENDOR spectra of ND2Y\(\text{Y731}^{+}/\alpha\) and ND2Y\(\text{Y731}^{+}/R_{411}\alpha\) \(\alpha\). 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 ND2\(\text{Y}^+\) Mims ENDOR spectra.\(^{12,33}\) However, we observe that the \(^3\)H hf tensor previously assigned to the moderately strong hydrogen bond between \(Y_{730}\) and \(Y_{731}\) in ND2\(\text{Y731}^{+}/\alpha\), which is almost perpendicular to the tyrosine ring plane,\(^{12}\) is absent in the ND2Y\(\text{Y731}^{+}/R_{411}\alpha\) \(\alpha\) spectrum. Therefore, the hydrogen bonding environment of NH\(\text{H2Y}^+\)/\(R_{411}\alpha\) \(\alpha\) 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 ND2\(\text{Y731}^{+}/R_{411}\alpha\) \(\alpha\) 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 (r_0.AH ~ 1.7–2 Å) to ND2_Y731/R411-A-z2 are observed, the ENDOR spectrum of ND2_Y731/R411-A-z2 exhibits a broad and structured matrix line, which is associated with weak hf interactions of the radical with distant nuclei(14) (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 ND2_Y731/R411-A-z2 is reminiscent of the one previously observed for ND2_Y356-b2, also located at the subunit interface and likely surrounded by a defined hydrogen bonded network of water molecules(13). The similarity between the ENDOR spectra of ND2_Y356-b2 and ND2_Y731/R411-A-z2 suggests a similar origin for the g values in these two mutants, which is distinct from that in ND2_Y731-z2. As noted above, in the case of ND2_Y356-b2 the g value was also strongly shifted (NH2_Y356: g_x = 2.0049 vs. free NH2-Y; g_x = 2.0061 (ref. 33)). Therefore, we propose that the g_x shift in NH2_Y731/R411-A-z2, as well as in ND2_Y356-b2, 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 NH2_Y731-z2 resulted in similar g-shifts. Overall, these data clearly indicate that the molecular orientation of ND2_Y731/R411-A-z2 is different to that of ND2_Y731-z2 and is affected by R411-A-z2 substitution.

PELDOR gives evidence for a conformational change in ND2_Y731/R411-A-z2

Our previous PELDOR studies(26) have demonstrated that half site reactivity of *E. coli* RNR allows for the detection of the diagonal inter-spin distance between Y122_r and any radical trapped in the second zβ pair (Fig. 4A).25,62 To gain insight into the location of NH2_Y731/R411-A-z2, 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(64–66) (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 zβ2 complex,25,62 suggesting more conformational heterogeneity for ND2_Y731/R411-A-z2, consistent with the observed flexibility of this residue. Nevertheless, the results clearly indicate that the R411 mutation induces a conformational change of ND2_Y731 to 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 ND2_Y731-z2.26 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.62 To confirm our assignment, we recorded PELDOR experiments at higher temperature (50 K), in which the Y122-z2 contribution to the re-focused echo is filtered and ND2_Y731-z2 is the only radical species detected (Fig. S9†). However, Y122-z2 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’-ND2_Y731 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 conformation of NH2_Y731/R411-A-z2 and the role of R411, we examined the available X-ray structures of *E. coli* z2s in the R411 region. In the structure of *E. coli* NH2_Y730-z2 (2XO4),28 Y731 is flipped away from NH2_Y309 as shown in Fig. 5. This altered conformation is compared with a second z 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 NH2_Y731 must rotate away from Y730 toward the z2 subunit, as observed for Y731 in the *E. coli* Y730NH2-Y-z2 structure (Fig. 5).
This reorientation is also supported by the ENDOR data, which indicate that the stacked conformation between NH$_2$Y$_{731}$ and Y$_{730}$ with a shared, perpendicular hydrogen bond is absent in NH$_2$Y$_{731}$/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$2 subunit interface. The exposure of NH$_2$Y$_{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$_2$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 2 Å. We note that the “flipped” conformation has not been observed in the single mutant NH$_2$Y$_{731}$/R$_{411}$A-$\alpha$2 or in the double mutant NH$_2$Y$_{731}$/Y$_{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 validated computational methods.

Fig. 4 (A) Diagonal distance between Y$_{122}$’ in $\beta$2 and ND$_2$Y$_{731}$/R$_{411}$A-$\alpha$2. (B) The ESE spectrum of ND$_2$Y$_{731}$/R$_{411}$A-$\alpha$2 is composed of the Y$_{122}$’-$\beta$2 spectrum (dashed and dotted black line) and the ND$_2$Y$_{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 $\pi$ 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$_2$Y-$\alpha$2 structure (2X04)† in green shows the reoriented Y$_{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.
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 NH3Y731/R411A 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/MB and DFG-SPP 1601, the Max Planck Society and NIH (GM29595 to JS).

Conclusions

This study has revealed that the E. coli RNR double mutant NH3Y731/R411A–2 unmaps 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 Y731 and Y730, and thus in the facilitation of collinear 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 Y731–α2 and Y356–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 Y731–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).

References
