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Function of the diiron cluster of *Escherichia coli* class Ia ribonucleotide reductase in proton-coupled electron transfer

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Abstract

The class Ia ribonucleotide reductase (RNR) from *Escherichia coli* (Ec) employs a free-radical mechanism, which involves bidirectional translocation of a radical equivalent or “hole” over a distance of ∼35 Å from the stable diferric/tyrosyl-radical (Y\(_{122}^•\)) cofactor in the β subunit to cysteine 439 (C\(_{439}\)) in the active site of the α subunit. This long-range, inter-subunit electron transfer occurs by a multi-step “hopping” mechanism via formation of transient amino acid radicals along a specific pathway and is thought to be conformationally gated and coupled to local proton transfers. Whereas constituent amino acids of the hopping pathway have been identified, details of the proton-transfer steps and conformational gating within the β subunit have remained obscure; specific proton couples have been proposed, but no direct evidence has been provided. In the key first step, the reduction of Y\(_{122}^•\) by the first residue in the hopping pathway, a water ligand to Fe\(_1\) of the diferric cluster was suggested to donate a proton to yield the neutral Y\(_{122}\). Here we show that forward radical translocation is associated with perturbation of the Mössbauer spectrum, especially the quadrupole doublet associated with Fe\(_1\). Density functional theory (DFT) calculations verify the consistency of the experimentally observed perturbation with that expected for deprotonation of the Fe\(_1\)-coordinated water ligand. The results thus provide the first evidence that the diiron cluster of this prototypical class Ia RNR functions not only in its well-known role as generator of the enzyme’s essential Y\(_{122}^•\), but also directly in catalysis.

Introduction

Ribonucleotide reductases (RNRs) catalyze the reduction of ribonucleotides to deoxyribonucleotides in all organisms, thereby providing and controlling the only de novo
pathway to the four precursors required for DNA replication and repair.\textsuperscript{1,2} RNRs use a free-radical mechanism, in which a transient cysteine thyl radical (C•) in the active site of the enzyme initiates substrate reduction by abstraction of a hydrogen atom (H•) from C3′.\textsuperscript{1,3-9} In class Ia RNRs, such as the RNR from aerobically growing \textit{Escherichia coli} (Ec), a stable tyrosyl radical (Y\textsubscript{122•}) in the Ec ortholog) in close proximity to a μ-oxo-(Fe\textsuperscript{III})\textsubscript{2} cluster in the β subunit\textsuperscript{10-14} reversibly generates the transient C• in the active site of the enzyme’s α subunit in the functional α\textsubscript{2}β\textsubscript{2} complex.\textsuperscript{5-7,15,16} A model of the complex, constructed by computer docking of the structures of the individual subunits\textsuperscript{7} and subsequently validated by electron-electron double resonance spectroscopic experiments,\textsuperscript{17-19} suggests a distance of ∼35 Å between Y\textsubscript{122•} in β and the H•-abstracting C\textsubscript{439} in α. Electron transfer (ET) between C\textsubscript{439} and Y\textsubscript{122} by a single tunneling step over that distance would be far too slow to account for the enzyme’s turnover rate (2-10 s\textsuperscript{-1}).\textsuperscript{15,20} Instead, this long-range inter-subunit ET is mediated by a chain of strictly conserved aromatic amino acids, which form transient radicals in a “hopping” mechanism (Scheme 1A).\textsuperscript{7,15,21-28} Direct detection of these pathway radicals in the wild-type enzyme has been hampered by a preceding rate-limiting conformational change within the α\textsubscript{2}β\textsubscript{2} complex.\textsuperscript{20} This slow conformational change, which occurs upon binding of substrate and allosteric effector to α and allows for translocation of the radical from its resting location on β-Y\textsubscript{122} to where it functions in catalysis on α-C\textsubscript{439}, masks the subsequent, fast chemical events.\textsuperscript{20} Substitution of pathway tyrosines by unnatural amino acids with altered redox properties led to the first detection of pathway radicals and provided the most direct evidence that these residues are redox-active “stepping stones” in the long-range ET (Scheme 1A).\textsuperscript{21-26} The individual ET hopping steps in the overall 35-Å hole-translocation process are thought to be coupled to multiple short-range proton transfer steps (i.e., proton-coupled ET or PCET), which effectively tune the thermodynamics of the component steps for efficiency and reversibility of the overall process.\textsuperscript{15,20} The coupling of ET to proton transfer (PT) steps could therefore permit radical translocation to be controlled by the PT steps, which could, due to their more stringent distance and orientation requirements, be controlled by the conformation of the protein: engagement of a proton-transfer pathway upon substrate binding could be the basis for the conformational gating in RNR.

Whereas the radical-hopping nature of the process and the identities of the mediating residues have (with the exception of tryptophan 48 in β, which may or may not be a mediator) been established, much less is known about the details of the proton transfers. It has been proposed that PT proceeds orthogonally to ET in the β subunit and collinearly in α.\textsuperscript{15,20} A recent electron-nuclear double resonance (ENDOR) spectroscopic study provided evidence for hydrogen bonds among the three ET pathway residues of α in the active α\textsubscript{2}β\textsubscript{2} complex, consistent with collinear PCET.\textsuperscript{30} Within the β subunit, specific proton coupling partners have been proposed (Scheme 1A), but little experimental evidence has been provided.\textsuperscript{27,31} Specifically, in the first step of forward (β-Y\textsubscript{122•} → α-C\textsubscript{439}) radical translocation, the neutral Y\textsubscript{122•} is reduced to the neutral Y\textsubscript{122} by β-Y\textsubscript{356} (or perhaps β-W\textsubscript{48}) in the pathway, and it has been suggested that the proton required to maintain neutrality of the Y\textsubscript{122} side chain is delivered orthogonally to ET by the water ligand on the iron ion in site 1 (Fe\textsubscript{1}).\textsuperscript{15,32} In this study, we have trapped the cofactor in β in its product state of the forward radical translocation process by using either the radical-trapping substrate analog 2′-azido-2′-deoxyuridine 5′-diphosphate (N\textsubscript{3}UDP) with the wild-type enzyme or a natural substrate (CDP) with a variant of the α-subunit containing the radical-stabilizing unnatural amino acid 3-aminotyrosine (NH\textsubscript{2}-Y) at the subunit-interfacial pathway residue α-Y\textsubscript{731} (Scheme 1B and C). We show that the Mössbauer spectrum of the (Fe\textsuperscript{III})\textsubscript{2} cluster, especially the quadrupole splitting parameter (ΔE\textsubscript{Q}) associated with Fe\textsubscript{1}, changes while the oxidation state of the cluster remains unchanged and that this spectral perturbation is specific to the form of the enzyme that has engaged in forward radical translocation. The nature of the observed perturbation – a ∼ 0.5 mm/s diminution in [ΔE\textsubscript{Q}] of Fe\textsubscript{1} with much smaller changes
to $|\Delta E_Q|$ of Fe$_2$ and the isomer shifts ($\delta$) of both sites – agrees remarkably well with the effect predicted by simple DFT calculations for removal of a proton from the Fe1-OH$_2$ ligand. The results provide the first direct evidence that the diiron cluster of the prototypical class Ia RNR from Ec not only serves its well-known role as generator of the Y$_{122^*}$

but also actively participates in the enzyme's catalytic cycle.

**Results and Discussion**

To initiate forward radical translocation and trap the enzyme in the product state of this step, the $\alpha$ and $\beta$ subunits were incubated in the presence of the positive allosteric effector, thymidine triphosphate (TTP), with the substrate analog N$_3$UDP, which brings about the irreversible reduction of Y$_{122^*}$ in $\beta$ along with the formation of a meta-stable, nucleotide-based, nitrogen-centered radical (N*) in the active site of $\alpha$ (Scheme 1B).

Conversion of $\beta$-Y$_{122^*}$ to the N* was confirmed by comparison of the X-band EPR spectrum at 14 K of the reaction sample (Fig. 1 A, red) to that of a control sample from which N$_3$UDP was omitted (Fig. 1 A, green). Subtraction of the features of the unreacted $\beta$-Y$_{122^*}$ from the spectrum of the N$_3$UDP-treated sample yields the spectrum of the N* (Fig. 1A).

Its intensity accounts for $\sim 36\%$ of the Y$_{122^*}$ originally present in the control ($-$ N$_3$UDP) sample. Conversions of $\leq 50\%$ have generally been observed in such experiments with Ec RNR and have been attributed to the facile reaction of only one of the $\alpha$-$\beta$ pair in the $\alpha$-$\beta$ hetero-tetramer ("half-of-sites reactivity"), a property thought to be intrinsic to the enzyme.

The spectra of control samples containing $\beta$, effector, either CDP or N$_3$UDP, and an $\alpha$ variant having the hopping pathway disabled by substitution of the subunit-interfacial Y$_{731}$ in $\alpha$ with F lack these new features and are essentially identical to the spectrum of the sample with wild-type $\alpha$ and $\beta$ before reaction with N$_3$UDP (Fig. S2). The spectrum of an additional control sample, in which the Y$_{122^*}$ in $\beta$ was reduced in the absence of $\alpha$ with hydroxyurea (HU) to yield the inactive (Fe$^{III}$)$_2$/Y$_{122^*}$ met form (Fig. S2), is also very similar to that of the active form. These results imply that the observed perturbation to the
spectrum of the (Fe\textsuperscript{III})\textsubscript{2} cluster in the wild-type enzyme caused by N\textsubscript{3}UDP is related to radical translocation and not to either the absence of Y\textsubscript{122}\textsuperscript{*} per se or nucleotide binding events.

The two quadrupole doublets that make up the spectrum of the radical translocation product were unambiguously assigned to Fe\textsubscript{1} and Fe\textsubscript{2} by site-selective labeling of \(\beta\) with \(^{57}\text{Fe}\) and \(^{56}\text{Fe}\). The two sites in \(Ec\) \(\beta\) have different affinities for Fe\textsuperscript{II}, and this property can be exploited to obtain \(\beta\) with the Mössbauer active \(^{57}\text{Fe}\) enriched in one or the other site.\(^{41}\) The spectrum of a sample of the complex prepared with \(\beta\) subunit having site 1 enriched with \(^{57}\text{Fe}\) can be simulated with the same parameters used for spectra of the uniformly \(^{57}\text{Fe}\) labeled samples above, and the relative intensities of the two quadrupole doublets indicate that \(\sim 73\%\) of the \(^{57}\text{Fe}\) resides in site 1 and \(\sim 27\%\) in site 2 (Fig. 1C, I, red and blue). Treatment of this sample with N\textsubscript{3}UDP yielded a \(\beta\)-Y\textsubscript{122}\textsuperscript{*} to N\textsuperscript{*} conversion of \(\sim 38\%\), as determined by EPR spectroscopy (Fig. S3, Table S2), similar to that achieved with uniformly \(^{57}\text{Fe}\)-labeled complex. The Mössbauer spectrum of the radical translocation product, obtained after subtraction of the unreacted component (Fig. 1C, II and III), constitutes \(38\%\) of the active cofactor, in agreement with the EPR quantification. It can be simulated with the same parameters used for the spectrum of the product in the uniformly labeled complex (Fig. 1C, III, red and blue). Owing to the site-selective labeling, the two quadrupole doublets have different intensities and can therefore be unambiguously assigned to Fe\textsubscript{1} (red) and Fe\textsubscript{2} (blue). Samples with \(^{57}\text{Fe}\) enriched in site 2 confirm this assignment (Fig. 1D). These samples contain \(\sim 24\%\) of the \(^{57}\text{Fe}\) in site 1 and \(\sim 76\%\) in site 2 (Fig. 1D, I, red and blue). Reaction with N\textsubscript{3}UDP resulted in conversion of \(\sim 47\%\) of initial \(\beta\)-Y\textsubscript{122}\textsuperscript{*} to N\textsuperscript{*}, as quantified by EPR (Fig. S3, Table S2), and produced the Mössbauer spectrum shown in Fig. 1D, II. The Mössbauer spectrum of the radical translocation product, obtained after removal of the unreacted component (Fig. 1D, III) and accounting for \(52\%\) conversion of the active cofactor, can again be simulated with the same parameters used for the spectrum of the product with the uniformly and site-1-enriched complexes (Fig. 1D, III, red and blue). Assignment of the two quadrupole doublets to Fe\textsubscript{1} (red) and Fe\textsubscript{2} (blue) shows that the spectrum of Fe\textsubscript{1}, the site with the water proposed to be the proton donor, changes much more (|\(\Delta E_Q\)| decreases by 0.5 mm/s) than that of Fe\textsubscript{2} upon N\textsubscript{3}UDP-induced trapping of the radical translocation product.

In addition to the use of the radical-trapping substrate analog, N\textsubscript{3}UDP, the product of forward radical translocation can also be trapped by using an \(\alpha\) variant that contains an unnatural amino acid incorporated into the hopping pathway (Scheme IC). The radical produced from 3-aminotyrosine (NH\textsubscript{2}Y\textsuperscript{*}) has a reduction potential estimated to be \(\sim 190\) mV less than that of Y\textsuperscript{*}, causing the radical to reside on this unnatural residue during catalysis by \(Ec\) RNR variants.\(^{23,47}\) The variant \(\alpha\) protein having NH\textsubscript{2}Y in place of Y\textsubscript{731} is capable of nucleotide reduction, and, upon its incubation with the \(\beta\) subunit, the effector ATP and the CDP substrate, a NH\textsubscript{2}Y\textsuperscript{*} accumulates to \(\sim 50\%\) of the total spin in the sample.\(^{23}\) The dependence on the presence of nucleotides and \(\beta\), and the ability of this enzyme to catalyze deoxynucleotide production, show that the NH\textsubscript{2}Y\textsuperscript{*} forms by gated radical translocation in the functional holoenzyme complex. Translocation of the radical from \(\beta\) to \(\alpha\)-NH\textsubscript{2}Y\textsubscript{731} is expected to be accompanied by the same change to the (Fe\textsuperscript{III})\textsubscript{2} cluster observed above in the wild-type complex upon reaction with N\textsubscript{3}UDP. Indeed, the experimental Mössbauer spectrum after reaction of \(\beta\) with \(\alpha\)-Y\textsubscript{731}NH\textsubscript{2}Y, ATP, and CDP and the product spectrum (45\% conversion) obtained after removal of the unreacted component (Fig. 2) are almost identical to the spectra of the radical translocation product trapped by N\textsubscript{3}UDP (Fig. 1B). The observation of the same perturbed quadrupole-doublet spectrum in samples in which the Y\textsubscript{122}\textsuperscript{*}-reduced \(\beta\) was trapped by either N\textsubscript{3}UDP or the \(\alpha\)-Y\textsubscript{731}NH\textsubscript{2}Y variant, but not in samples prepared either with a pathway-disabled \(\alpha\) or by reduction of Y\textsubscript{122}\textsuperscript{*} by HU in the absence of \(\alpha\), strongly suggests that the change to the (Fe\textsuperscript{III})\textsubscript{2} cluster is associated

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specifically with functional translocation of the radical from Y_{122}^• into the hopping pathway.

To evaluate whether the change to the Mössbauer spectrum of the diferric cluster observed upon radical translocation is consistent with the proposed deprotonation of the Fe$_1$-OH$_2$ (Scheme 1B and C), we performed a series of DFT calculations. Remarkably, the calculations, performed by the broken-symmetry DFT methodology,$^{48}$ predict the same qualitative change to the Mössbauer parameters upon removal of a proton from the Fe$_1$-OH$_2$ [0.47-0.74 mm/s decrease in $|\Delta E_Q|$ for Fe$_1$, a much smaller (≤0.25 mm/s) increase or decrease in $|\Delta E_Q|$ of Fe$_2$, and almost no change (0.04 mm/s) to the isomer shift of either site; Fig. 3 and Table S3] as is observed experimentally upon radical translocation. The calculations were performed by starting from the published high-resolution structure of the Ec β protein,$^{49}$ which is purportedly of the met form. All first-sphere (ligand) residues and non-protein oxygen ligands [an HO(H) ligand to each iron and the oxo bridge] were included in the models (Fig. S4). Two sets of models were considered. One set includes both the diferric cluster and the radical tyrosine (Y$_{122}$) in either the resting state of the cofactor (Fe$_1$-OH$_2$/Y•) or the postulated radical-translocation-product state (Fe$_1$-OH/Y). A second set includes the diferric cluster in either its resting (Fe$_1$-OH$_2$) or radical translocation (Fe$_1$-OH) state but omits the tyrosine (Fig. S4). To limit the number of atoms while still preventing ligand motions that would be precluded by their attachment to the protein backbone from occurring during geometry optimization, the approach used by Roos and Siegbahn was adopted.$^{50}$ With these geometric constraints, none of the calculated models diverged markedly from the experimental structure during optimization (Fig. S4). In addition to calculations in the gas phase, the effect of the protein environment on the calculated Mössbauer parameters was evaluated using the COSMO solvation model$^{51,52}$ with various dielectric constants ($\epsilon$) of 4, 10, and 40. Calculations using $\epsilon = 4$ reproduce the experimental parameters remarkably well, to within ±0.18 mm/s for the models that include Y$_{122}$ (Fig. 3A). Moreover, all the calculations, whether in the gas phase or with the COSMO solvent model and with Y$_{122}$ included or omitted, reproduce the essential features of the experimental spectral perturbation, giving a relatively large decrease in $|\Delta E_Q|$ and much smaller change in $\delta$ for site 1 and small changes in $|\Delta E_Q|$ and $\delta$ for site 2 (Fig. 3B and Table S3). The results imply that the observed effect arises directly from the change in the charge of the Fe$_1$ HO(H) ligand rather than some interaction of the cluster with the Y$_{122}$/Y$_{122}^•$. Consistent with this conclusion and the experimental Mössbauer spectra, parameters calculated for a model having the reduced, neutral Y$_{122}$ and the Fe$_1$-OH$_2$ ligand (corresponding to the met form of the protein) do not deviate significantly from those calculated for the active state (with Y$_{122}^•$ and Fe$_1$-OH$_2$). These DFT calculations thus establish that the change to the Mössbauer spectrum associated with radical translocation is consistent with the proposed donation of a proton to Y$_{122}$ by the Fe$_1$-OH$_2$ in the first PCET step.

We anticipated that the Fe$_1$-OH radical translocation product would be meta-stable and eventually undergo protonation (with the ultimate source being bulk solvent) to generate the Fe$_1$-OH$_2$ species of the stable met form (Fe$_1$-OH$_2$/Y). To test this notion, the radical translocation product trapped with N$_3$UDP was thawed and incubated on ice to permit decay of the N• and enable subsequent adjustments of the protein complex and the (Fe$^{III}$)$_2$ cluster. Periodically, the sample was re-frozen for acquisition of its Mössbauer spectrum. Spectra acquired after total incubation times of 10 to 260 minutes demonstrate the return of the spectrum of the resting (presumably Fe$_1$-OH$_2$) form of the cluster (Fig. 4). This result further confirms that the observed perturbation to the (Fe$^{III}$)$_2$ cluster upon reaction with N$_3$UDP or Y$_{731}$NH$_2$Y-α and CDP is specific to the complex actively engaged in radical translocation and catalysis. The regeneration is relatively slow ($t_{1/2}$ of ~60 min at ~0°C), consistent with the hypothesis that it reflects the slow diffusion of an extra proton from bulk.
solvent to the Fe$_1$–OH in the protein interior to convert it to Fe$_1$–OH$_2$ of the resting met form (perhaps subsequent to decay of the N• and disengagement of β from α with a $t_{1/2}$ of 23 min at 25 °C [53]). That this proton transfer from bulk solvent would be slow is supported by studies in which the kinetics of electrochemical reduction of the β subunit in the presence of a facile ET mediator (methyl viologen) were monitored. These experiments revealed that the (Fe$^{III}$)$_2$ center in the met form can be reduced relatively rapidly (within seconds), whereas reduction of the active (Fe$^{III}$)$_2$/Y$_{122}$• form of the protein proceeds with the initial fast reduction of Y$_{122}$• followed by much slower reduction of the (Fe$^{III}$)$_2$ cluster. The observations suggest that the cluster in the met form and the one generated from the active (Fe$^{III}$)$_2$/Y$_{122}$• cofactor upon rapid Y$_{122}$• reduction are somehow different. A reasonable explanation is that the cluster after fast Y$_{122}$• reduction has the same number of protons as the radical translocation product, possessing the Fe$_1$–OH generated by transfer of a proton from the Fe–OH$_2$ to Y$_{122}$ (Scheme 1B and C), whereas the stable met form produced by HU reduction of Y$_{122}$• in the absence of α has the cluster in the Fe$_1$–OH$_2$ form. The absence of this proton in the initial Y$_{122}$•-reduced β and presence in the stable met form would make the charge of the buried cluster different by one unit, altering the electrostatics of the site and potentially causing the difference in reduction kinetics of the two forms.

Conclusion

The diiron cluster in class Ia Ec RNR has long been known to generate the Y$_{122}$• in the initial activation of the β subunit by reaction of its (Fe$^{II}$)$_2$ form with O$_2$. Our results now strongly suggest that the (Fe$^{III}$)$_2$ cluster also actively functions in the catalytic cycle, specifically during translocation of the oxidizing equivalent or hole from its resting position on Y$_{122}$• in β to the nucleotide reduction site in α. Reduction of Y$_{122}$• upon forward radical translocation requires transfer of a proton to yield a neutral Y$_{122}$, and, upon reverse radical translocation and reoxidation to Y$_{122}$•, the proton should be returned. The Mössbauer-detected change to the cluster seen upon use of either the substrate analog or the α variant is almost certainly associated specifically with propagation of the radical into the hopping pathway, because the perturbation (1) is observed when the radical on Y$_{122}$ translocates in a functionally relevant reaction into α, but not when it is reduced in a nonfunctional context by HU, (2) relaxes upon decay of the N• in α, and (3) is not observed in a complex having the hopping pathway blocked by the α-Y$_{731}$F substitution. The nature of the spectral perturbation (significant decrease in $|\Delta E_Q|$ of site 1 and much smaller changes to the other three parameters) implies that the oxidation state of the cluster does not change and matches that predicted by DFT calculations for removal of a proton from the Fe$_1$–OH$_2$ ligand. Our data are thus consistent with the previous suggestion that this water ligand serves as the proton-coupling partner to Y$_{122}$ for radical translocation (Scheme 1). The reduction of Y$_{122}$• constitutes the first step of forward radical translocation and might, therefore, be a key step in the gating of the process by the protein. If one or more protein side chain is required to mediate this proton transfer (e.g., the nearby D$_{84}$ carboxylate ligand), a conformational change in the αβ$_2$ complex occurring upon substrate binding could engage this proton-transfer pathway and thereby open the gate to reduction of Y$_{122}$• by either W$_{48}$ or Y$_{356}$ in the initial step of the long-distance radical translocation.

Methods

Materials, protein production and purification, reconstitution of RNR-β with $^{57}$Fe and/or $^{56}$Fe, and activity assays are described in the Supporting Information.
Reactions with N$_3$UDP

The reaction was carried out in a final volume of 0.6 mL and contained 0.3 mM $\alpha_2$ (or 0.29 mM $\beta_2$ in the case of uniformly $^{57}$Fe labeled $\beta_2$ and 0.29 mM $\beta_2$ in the case of Y$_{731}$F-$\alpha_2$), 0.8 mM TTP, 1 mM N$_3$UDP, 15 mM MgSO$_4$, 1 mM EDTA, and 1 mM DTT in HEPES buffer. The reaction was initiated by the addition of N$_3$UDP and $\beta_2$ and allowed to proceed at RT (21 ± 2 °C) for a total reaction time of 2.5 min. Aliquots (0.3 mL) were transferred to Mössbauer and EPR sample cells and frozen in liquid N$_2$.

Reaction of Y$_{731}$NH$_2$Y-$\alpha_2$ with substrate

Pre-reduced Y$_{731}$NH$_2$Y-$\alpha_2$ and $^{57}$Fe reconstituted $\beta$ in assay buffer were concentrated to 0.3 mL, and mixed with an aliquot (0.05 mL) of ATP and CDP in assay buffer. The final reaction solution (0.35 mL) contained 0.25 mM Y$_{731}$NH$_2$Y-$\alpha_2$, 0.25 mM $\beta_2$, 3 mM ATP and 1 mM CDP and was incubated at RT (22°C) for a total reaction time of 20 s and frozen in liquid N$_2$.

EPR spectroscopy and analysis

EPR spectra were recorded on a Bruker ESP300 spectrometer equipped with a ER 041 MR Microwave Bridge and a Bruker 4102ST TE$_{102}$ X-band resonator. Spectrometer configuration and data acquisition was performed by an external PC via GPIB interface using EWWIN 6.1 software from Scientific Software Services. Spectra were acquired at a temperature of 14.0 ± 1 K, a microwave power of 8 $\mu$W a microwave frequency of 9.45 GHz, a modulation amplitude of 3 G, a modulation frequency of 100 kHz, a receiver gain of $5 \times 10^4$, and a conversion time of 0.029 s. Four scans were averaged for the spectra acquired from 2,000 to 4,000 G. For spectra collected over the narrower field range from 3,180 to 3,580 G, a modulation amplitude of 1.0 G was used, and two scans were averaged.

The total electron spin concentration in each sample was determined by integrating its EPR absorption spectrum and comparing the integrated area to that of the spectrum of a standard containing 1.025 mM CuSO$_4$, 2 M NaClO$_4$, and 0.01 M HCl. The first derivative spectra recorded from 2,000 to 4,000 gauss G were integrated and the baselines were corrected by using a linear function. The second integral was formed and integrated areas were corrected for differences in $g$-values, as previously described.

Samples treated with N$_3$UDP contain a mixture of Y$_{122}^*$ and the nitrogen-centered radical (N$^\ast$). Removal of the features of the Y$_{122}^*$ by subtraction of the appropriately scaled spectrum of the control (− N$_3$UDP) sample yields the spectrum of the N$^\ast$. Quantification of the subspectra of Y$_{122}^*$ (scaled control spectrum) and N$^\ast$ in the samples was accomplished by integrating the two subspectra, applying a linear baseline correction, and forming the second integral to determine the area. These areas were used to calculate the percent area of the two subspectra, which were then multiplied by the measured total spin concentration of the samples to determine the concentration of each radical. The values are reported in Table S2. The concentrations of the Y$_{122}^*$ and N$^\ast$ in the N$_3$UDP treated samples can also be calculated using the scaling factor of the Y$_{122}^*$ control spectrum that was subtracted. This scaling factor was multiplied by the spin concentration of the control sample to determine the concentration of Y$_{122}^*$ in the sample. This was then subtracted from the concentration of the reacted sample to determine the remaining spin concentration, which was attributed to N$^\ast$. The values obtained by this procedure agree well with the values using the double integral of the two subspectra reported in Table S2.
Mössbauer spectroscopy and analysis

Mössbauer spectra were recorded at a temperature of 4.2 K and an externally applied magnetic field of 53 mT oriented parallel to the γ-beam on a SVT-400 spectrometer from WEB Research (Edina, MN). Data analysis was performed using WMOSS (WEB Research, Edina, MN). Isomer shifts are quoted relative to the centroid of a spectrum of a metallic foil of α-Fe at room temperature.

Parameters of the spectrum of the active α₂β₂ complex before reaction—The Mössbauer spectrum of the active α₂β₂ complex consists of two symmetrical quadrupole doublets of equal intensity. The spectrum was fitted by two quadrupole doublets with the linewidths (Γ) of the doublets constrained to be the same. The resulting parameters are cited in the main text and in Table S1.

Analysis of the spectrum of the α₂β₂ complex after reaction with N₃UDP—Removal of the contribution of the unreacted (Fe³⁺)₂ cluster (71% of total Fe of the uniformly ⁵⁷Fe labeled sample) from the spectrum of the sample reacted with N₃UDP resolves the spectrum of the radical translocation product. The subtraction was guided by visual inspection of the resolved left line at -0.76 mm/s and by the objective of making the resulting spectrum symmetric. The spectrum of the radical translocation product was then analyzed as two symmetrical quadrupole doublets of equal intensity and linewidth. Two sets of physically meaningful parameters (left-right solution and inner-outer solution, Table S1) fit the data equally well and cannot be distinguished owing to the 1:1 ratio of the two quadrupole doublets.

The dependence of the Mössbauer parameters obtained for the radical translocation product on the fraction of the spectrum of unreacted complex subtracted from the experimental spectrum was evaluated. We found that δ and ΔE_Q vary by only 0.001-0.02 mm/s, which is within the intrinsic uncertainty limit for the parameters (0.02 mm/s), when reference spectra for the radical translocation product corresponding to 29 ± 3% of total Fe (71 ± 3% of the experimental spectrum attributed to the unreacted cluster and removed in the subtraction) were analyzed.

Analysis of the spectra with site selective ⁵⁷Fe labeled β—To determine the occupancies of ⁵⁷Fe in sites 1 and 2 in the site-selectively labeled βs, Mössbauer spectra of the ⁵⁷Fe₁ and ⁵⁷Fe₂ enriched β (1.3 mM β₂) used in all the experiments were recorded. The spectra were simulated by fixing the parameters to those obtained from analysis of the spectrum of the uniformly ⁵⁷Fe labeled β in the sample of the α₂β₂ complex and allowing only the areas of the two doublets to vary. The relative areas for the two doublets correspond to the relative amounts of ⁵⁷Fe in sites 1 and 2, respectively. These occupancies were kept constant for all subsequent analyses of the spectra of reaction samples.

Subtractions of the unreacted component (scaled spectra of α₂β₂ before reaction) from the spectra after reaction with N₃UDP to obtain the spectrum of the radical translocation products were carried out as described above for the uniformly ⁵⁷Fe labeled samples. The product spectrum was simulated with the same parameters as used for the spectrum of the product in the uniformly labeled complex but using the areas for the two quadrupole doublets determined above. To evaluate the robustness of the percentage of intensity assigned to the unreacted complex, theoretical spectra for the two quadrupole doublets of the radical translocation product (using the quoted parameters) were subtracted in appropriate fractions from product spectra generated by subtraction of different amounts of the unreacted component.
The spectra of the site-selectively labeled samples allow unambiguous assignment of the two quadrupole doublets to Fe$_1$ (red) and Fe$_2$ (blue). In addition, they should, in principle, allow differentiation between the two possible sets of parameters (left-right solution and inner-outer solution, Table S1). However, the parameters of the two solutions are very similar, and we cannot make this assignment with confidence. While the two low-energy lines are at least partially resolved, both solutions (inner-outer and left-right) assign the -0.52 mm/s and -0.31 mm/s lines to the quadrupole doublets associated with Fe$_1$ and Fe$_2$, respectively. For the high-energy lines, the two lines are nearly at the same position (1.37 mm/s and 1.45 mm/s). The small difference in line position of only of 0.08 mm/s precludes a distinction between the two solutions, because variations in the linewidth influence the simulations to a greater extent than the choice of inner-outer versus left-right parameters.

DFT calculations

All geometry optimizations were performed using the GAUSSIAN 09$^{56}$ revision C.01 package. The initial geometry guess was based on the crystal structure of Ec RNR β, which is purportedly the diferric met form (PDB 1MXR$^{49}$). First shell residues around the irons and Y$_{122}$ were cut at the α carbon. To mimic the strain imposed by the protein environment, the α carbon and two out of the three adjacent hydrogen atoms were frozen in all calculations, similarly to the procedures described by Roos and Siegbahn.$^{32,50}$ Four different models were explored: the active resting state (Fe$_1$-OH$_2$/Y•) and the predicted radical translocation product (Fe$_1$-OH/Y) of the cofactor, both with and without Y$_{122}$ to account for the tyrosine’s influence on the Mössbauer parameters. In addition, the met form (Fe$_1$-OH$_2$/Y) of the enzyme was modeled with Y$_{122}$ and calculated for reference. Calculations were performed in the gas phase as well as in three distinct dielectric environments implemented via a conductor-like screening model (COSMO)$^{51}$ in the polarizable continuum model (PCM)$^{52}$ framework, termed as C-PCM, with ε = 4, 10 and 40. Optimizations were performed within the unrestricted DFT formalism with the three-parameter Becke–Lee–Yang–Parr (B3LYP) hybrid functional.$^{57,58}$ The antiferromagnetic coupling between the irons was achieved using broken-symmetry methods based on the ones developed by Noodleman et al.$^{48}$ Pople’s 6-31g basis set$^{59}$ was used on all atoms except the irons, which were represented with the 6-311+g* basis set. Calculations of spectroscopic parameters were performed using the optimized geometries with the unrestricted DFT formalism using the ORCA$^{60}$ 2.9.1 package with the B3LYP functional and the 6-311g* basis set on all atoms. In the case of the iron atoms, a diffuse function was added to the basis set (6-311+g*). The broken symmetry state was realized via the “flipspin” feature implemented in ORCA. All ORCA calculations utilized the resolution of the identity Coulomb density fitting approximation$^{61}$ with the chain of spheres exchange (RIJCOSX).$^{62}$ The $^{57}$Fe isomer shifts (δ) were calculated from the electron density at the iron nucleus ($\varrho(0)$) using linear correlation response theory.$^{53,64}$ Calibration of $\varrho(0)$ values was performed by correlating DFT calculated $\varrho(0)$ values of iron complexes using the geometries presented by Römelt et al. with the experimental isomer shifts of these complexes.$^{65}$ The linear fit yielded the equation $\delta = 0.0963 - 0.3857 \varrho(0) - 11616.5$ with δ in mm/s and $\varrho(0)$ in au$^{-3}$, and RMS = 0.9899 (Fig. S5).

Supplementary Material

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Acknowledgments

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References

Preparations of Ec β generally contain more (Fe$^{III}$)$_2$ clusters than Y$^{122}$•. Metal analysis and Y• quantification indicate that our preparation here contains 71% of active (Fe$^{III}$)$_2$/Y$^{122}$• and 29% of the reduced, inactive (Fe$^{III}$)$_2$/Y$^{122}$• (commonly referred to as “met”) form, consistent with published results. Details of this quantification are provided in Table S2 of the Supporting Information. All % conversions determined by analysis of the Mössbauer spectra are reported here relative to the active (Fe$^{III}$)$_2$/Y$^{122}$• form rather than the total (Fe$^{III}$)$_2$ cluster.


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Figure 1.
EPR and Mössbauer spectra of Ec RNR before and after N₃UDP-induced trapping of the product of forward radical translocation. (A) EPR spectra of uniformly $^{57}$Fe labeled $\alpha_2\beta_2$ with TTP and in the absence (green) or presence (red) of N₃UDP. Subtraction of the features of the unreacted $\text{Y}_{125}^+$ (green) from the spectrum of the N₃UDP-treated sample (red) yields the spectrum of the $\text{N}^+$ (blue). 4.2-K/53-mT Mössbauer spectra of uniformly $^{57}$Fe labeled (B), site 1 $^{57}$Fe enriched (C) and site 2 $^{57}$Fe enriched (D) $\alpha_2\beta_2$ with TTP and in the absence (I) or presence (II) of N₃UDP. The solid lines in I are simulations of the two quadrupole doublets (red and blue) of the resting (Fe$^{\text{III}}$)₂ cluster with parameters quoted in the text and the sum of the two doublets (black). The solid line in II is the spectrum from I scaled appropriately to remove the contribution of the unreacted cofactor, and subtraction of this contribution from II reveals the spectrum of the product of the forward radical translocation (III), which can be simulated with two quadrupole doublets (red and blue; parameters quoted in the text). The sum of the two quadrupole doublet simulations is shown as a black line. The spectrum in IV is the total difference of I-II and the solid line is a simulation of the difference spectrum as a sum of the four quadrupole-doublet components.
Figure 2.
4.2-K/53-mT Mössbauer spectra of the product of forward radical translocation trapped in the \((Y_{731}NH_2Y-\alpha)_2\beta_2\) complex. (I) Sample prepared with \(\beta, Y_{731}NH_2Y-\alpha,\) ATP and CDP. The green line is the scaled control spectrum of \(\beta\) that had been incubated with pathway-blocked \(Y_{731}F-\alpha,\) ATP and CDP, and subtraction of this spectral contribution reveals the spectrum of the product of forward radical translocation (II). The green line overlaid in II is the spectrum of the radical translocation product induced with N_3 UDP from Fig.1B, III, and is shown to illustrate the near identity of the perturbed spectra resolved by the two different trapping approaches. The spectrum in III is the total difference of the spectrum of \((Y_{731}F-\alpha)_2\beta_2\) in the presence of ATP and CDP subtracted from I, and the green line is the total difference spectrum from the \(\alpha_2\beta_2 \pm N_3 UDP\) experiment in Fig.1B, IV.
Figure 3.
Comparison of the experimental quadrupole splitting ($\Delta E_Q$) parameters for the resting and radical-translocation-product states of the cofactor to the values calculated by DFT. (A) Absolute values of $\Delta E_Q$ from experiment (gray bars) and the DFT calculations using a dielectric constant of $\varepsilon = 4$ for models of either the full cofactor including $Y_{122}$ (solid colored bars) or just the diferric cluster without $Y_{122}$ (striped colored bars). The blue bars correspond to Fe$_1$ and the red bars to Fe$_2$. (B) The changes in the absolute values of $\Delta E_Q$ associated with the radical-translocation/deprotonation event either from the experiments (dashed gray lines) or from the calculations on the models including (solid bars) or omitting (striped bars) $Y_{122}$. The color coding is the same as in panel A.
Disappearance of the spectrum of the N$_3$UDP-trapped radical translocation product and return of the spectrum of the resting (Fe$^{III}$)$_2$ cluster upon prolonged incubation on ice. 4.2-K/53-mT Mössbauer spectra of $\alpha_2 \beta_2$ with TTP and in the absence or presence of N$_3$UDP. The +N$_3$UDP sample was thawed, incubated on ice, and periodically re-frozen after total incubation times of 10 to 260 minutes for re-acquisition of its Mössbauer spectrum. The spectrum at $t = 260$ minutes is almost identical to the spectrum of $\alpha_2 \beta_2$ with TTP in the absence N$_3$UDP (overlaid as a solid line).
Scheme 1.
Proposed radical translocation pathway (A) and schematic representation of the two approaches used to trap the forward radical translocation product in Ec RNR (B) and (C). In (A) W₄₈ and D₂₃₇ are shown in gray because there is currently no direct evidence for involvement of W₄₈ in radical translocation. Trapping of the forward radical translocation product induced by N₃UDP in the wild-type α₂β₂ complex is shown in (B), and by CDP in a complex with an α variant containing the radical-stabilizing unnatural amino acid 3-amino-L-tyrosine (NH₂-Y) at residue α-Y₁₇₁ (C). EPR- and Mössbauer-observable species are illustrated in (B) and (C) by red and blue outlines, respectively.