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Redox-Linked Changes to the Hydrogen-Bonding Network of Ribonucleotide Reductase β2

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Supporting Information

ABSTRACT: Ribonucleotide reductase (RNR) catalyzes conversion of nucleoside diphosphates (NDPs) to 2′-deoxynucleotides, a critical step in DNA replication and repair in all organisms. Class-Ia RNRs, found in aerobic bacteria and all eukaryotes, are a complex of two subunits: α2 and β2. The β2 subunit contains an essential diferric–tyrosyl radical (Y122O$^\bullet$) cofactor that is needed to initiate reduction of NDPs in the α2 subunit. In this work, we investigated the Y122O$^\bullet$ reduction mechanism in *Escherichia coli* β2 by hydroxyurea (HU), a radical scavenger and cancer therapeutic agent. We tested the hypothesis that Y122OH redox reactions cause structural changes in the diferric cluster. Reduction of Y122O$^\bullet$ was studied using reaction-induced FT-IR spectroscopy and [^{13}C]aspartate-labeled β2. These Y122O$^\bullet$ minus Y122OH difference spectra provide evidence that the Y122OH redox reaction is associated with a frequency change to the asymmetric vibration of D84, a unidentate ligand to the diferric cluster. The results are consistent with a redox-induced shift in H-bonding between Y122OH and D84 that may regulate proton-transfer reactions on the HU-mediated inactivation pathway in isolated β2.

Reorganization of metal-bound carboxylates is proposed to play a significant role in modulating the reactivity of many non-heme proteins requiring diiron.[1] This reorganization may take the form of pronounced changes in metal ligation such as "carboxylate shifts" or more subtle changes in local hydrogen-bonding networks. The β2 subunit of class-Ia ribonucleotide reductase (RNR), having an essential diferric–tyrosyl radical (Y122O$^\bullet$) cofactor (Figure 1), provides an example in which these changes are proposed to occur during a number of catalytic processes. Despite the prevalence of these changes and their importance in catalysis, there are a limited number of ways to measure them experimentally. Reaction-induced FT-IR spectroscopy is unique among the biophysical methods for its ability to monitor subtle shifts in carboxylate ligands in real time. In this work, we utilize the inherent sensitivity of FT-IR spectroscopy to detect reorganization of carboxylate D84 in the β2 diferric–YO$^\bullet$ cluster, thus showcasing a powerful method for monitoring these important changes.

RNR catalyzes reduction of ribonucleotides to the corresponding deoxy forms and thus is a key participant in DNA biosynthesis and repair. It is composed of two subunits that form the active $(\alpha2)_n\beta2$ (n = 1, 3) complex.[2] In β2, carboxylate rearrangements/H-bonding changes may occur in three different scenarios (Figure 1). Carboxylate shifts are known to accompany O_2 activation of diferrous β2 during assembly of the active diferric–Y122O$^\bullet$ state (Figure 1A). During turnover (Figure 1B), Y122O$^\bullet$-β2 generates a cysteine radical, C439$^\bullet$, at the substrate binding site in α2 to initiate substrate reduction. This process involves reversible proton-coupled electron transfer (PCET) over 35 Å between Y122O$^\bullet$ and C439.[3] Nucleotide reduction is limited by a conformational gate[4] that may be regulated by proton transfer to Y122O$^\bullet$ from the metal cluster.[5] Thus, changes in H-bonding accompanying reversible Y122O$^\bullet$ reduction during catalysis are also predicted. Here we show that the asymmetric stretching vibration (ν_{as}) of a carboxylate ligand, D84, is a sensor for changes in H-bonding in the diiron cluster.

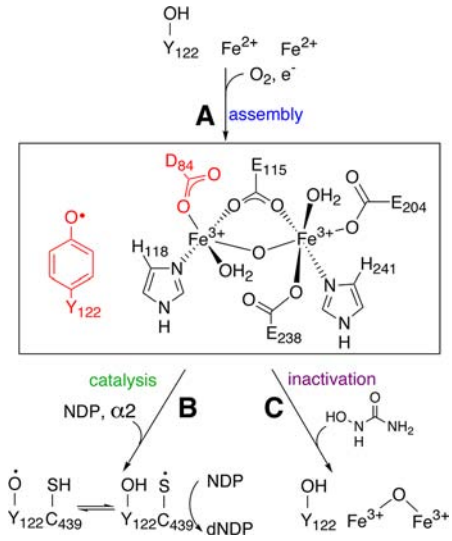

Figure 1. Possible scenarios for carboxylate reorganization in the diiron cofactor in *E. coli* class-Ia β2. (A) Active cofactor is first assembled by reacting diferrous β2, O_2, and reductant. (B) Stable Y122O$^\bullet$ is reduced concomitantly with C439 oxidation by reversible, long-range PCET during catalysis. (C) Alternatively, the cofactor may be rendered inactive by small-molecule-mediated reduction of Y122O$^\bullet$. We monitor changes to the amino acids shown in red upon HU-mediated inactivation.

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To achieve this aim, we employed hydroxyurea (HU) to reduce Y122O• (Figure 1C). HU was chosen to trigger single-electron reduction of the diferric−Y122O• cofactor to the diferric−Y122OH or "met" state [Figure S1 in the Supporting Information (SI)].[6] This process was monitored kinetically (Figure S2) by the loss in absorbance at 410 nm associated with Y122O•. The rate constant for Y122O• reduction in H_2O was 0.33 $M^{-1}s^{-1}$, similar to previously reported values.[7] The FT-IR experiments were conducted in D_2O buffers (see the SI); thus, the HU kinetics was also evaluated under these conditions (Figure S2A). Comparison of the second-order rate constants for Y122O• reduction obtained in 100% D_2O and 100% H_2O revealed a large solvent isotope effect (SIE = k_{H_2O}/k_{D_2O}) of 15±1 at 20 °C (k_{H_2O} = 0.33 $M^{-1}s^{-1}$; k_{D_2O} = 0.023 $M^{-1}s^{-1}$). Previous studies showed that the Y122O• reaction with HU is nonsaturable, suggesting that an initial, specific binding of HU to β2 is not required for radical reduction.[6b] While the origin of the large SIE is not known, it could be consistent with conformational gating,[5,8] simultaneous multiple proton transfers, and/or proton tunneling.[9]

The reaction-induced FT-IR technique was described previously.[10] Vibrational spectra were recorded during the HU-mediated reduction of Y122O•, and the Y122O• minus Y122OH difference spectra were generated (Figures 2A and S3). Isotope-based assignments of FT-IR spectral features are possible using double-difference spectra, called isotope-edited spectra (Figure 2A). Difference FT-IR data collected independently from natural abundance (NA) and specific isotopically labeled (e.g., ^{13}C) samples were subtracted (Figures 2A and S4) to identify vibrational bands whose amplitude and/or frequency changed upon incorporation of the heavier isotope. Previously, we used this approach with $^2H_4$-labeled Tyr to assign a positive band at 1498 cm^{-1} to the CO stretching vibration of Y122O• and a band at 1514 cm^{-1} to a ring stretching vibration of Y122OH.[10]

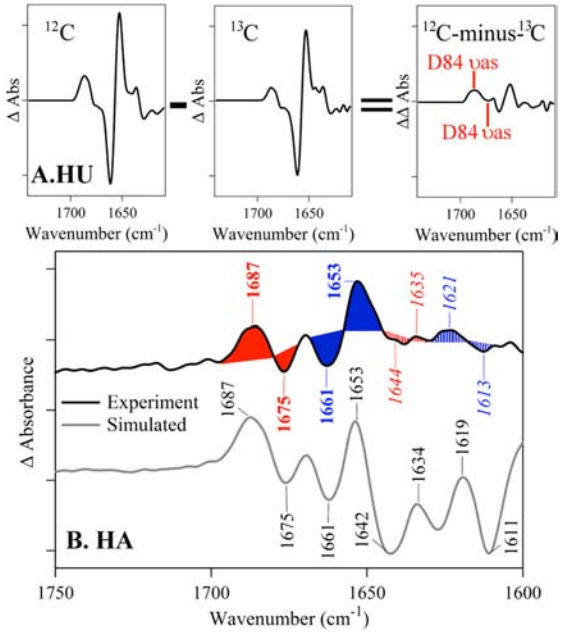

Figure 2. (A) Schematic of the isotope-edited FT-IR spectrum, reflecting [4-^{13}C]Asp (40%) labeling and reduction of Y122O• with HU. Band assignments to ν_{as} of D84 are labeled in red. (B) NA minus [U-$^{13}C_4$]Asp isotope-edited spectrum (top; also see Figures S3 and S4) for the HA reaction. The reaction mixture contained 100 μM β2 and 25 mM HA in 5 mM HEPES, pD 7.6 (20 °C, 50 μm spacer). Band assignments were established by spectral simulation (bottom). The bottom trace is the simulated isotope-edited spectrum, accounting for the data and produced from the NA spectrum in Figure S3F assuming ^{13}C shifts of −43 cm^{-1}. Red and blue labels represent assignments to D84 ν_{as} and amide-I bands, respectively. Bands labeled in bold (solid shading) and italics (dashed shading) represent NA and ^{13}C-labeled samples, respectively. Tick marks in (B) are 2 × 10^{-4} AU.

In addition to changes associated with Y122O• and Y122OH, the HU-derived difference spectra (Figure 2A) reflect other coupled changes in the environment of Y122OH, which may include structural changes at iron cluster ligands. D84 is a metal ligand, so its vibrational signature can easily be distinguished from those of free carboxylates and carboxylic acids.[11] Bands in the 1680−1670 cm^{-1} region were observed in the HU difference spectrum (Figure 2A). This region is expected to reflect frequency changes for unidentate carboxylate ligands.[11a] However, there is considerable variation in the expected frequencies of carboxylate ligands, depending on the metal oxidation state, ligand geometry, and H-bonding.

To identify the characteristic bands associated with H-bonding shifts at D84 unambiguously, we acquired the reaction-induced FT-IR spectrum associated with reduction of Y122O• and the iron cluster. All of the available X-ray structures predict that D84 loses its H-bonding to bound water molecules when the iron cluster is reduced (Figure S1). This loss of H-bonding and oxidation state change would be expected to alter the vibrational frequencies of D84, the only aspartate ligand to the iron cluster.[12]

To reduce both Y122O• and the iron cluster, hydroxylamine (HA) was employed. In H_2O, reduction of the diferric cluster by HA proceeds at a much lower rate than that of Y122O•.[13] However, in D_2O, the optical spectra (Figure S2C) provide the first evidence for the synchronized reduction of Y122O• (410 nm band) and the diferric cluster (325 and 370 nm bands) by HA. Like the HU reaction, the reaction with HA occurs on the time scale of the reaction-induced FT-IR measurement. Thus, the HA spectrum contains contributions from both the iron cluster and Y122O• reductions.

To assign bands to D84 in the HA spectrum, an isotope-edited spectrum was created, through the use of a [U-$^{13}C_4$]Asp isotopologue of β2 (Figure 2B top). The [U-$^{13}C_4$]Asp sample was produced in an Asp auxotroph and was ∼40% labeled (see the SI). The isotope-edited reaction-induced FT-IR spectrum, which identifies all Asp vibrational modes involving carbon displacement, is shown as the top spectrum in Figure 2B. In the region presented, the expected contributions arise from the ν_{as} normal mode of the Asp side chain and the amide-I (peptide C═O) band of the Asp amide bond. In Figure 2B top, bands of NA Asp are positive in the Y122O• state and negative in the Y122OH state. Isotope-shifted bands appear with opposite signs.

In the HA isotope-edited spectrum, two bands were observed at (+) 1687 and (−) 1675 cm^{-1} (red solid-shaded peaks in Figure 2B top). Because these bands were observed in the isotope-edited spectrum, they are assignable to D84. To predict the ^{13}C isotope shifts for these bands, a simulated isotope-edited spectrum was generated assuming the expected 43 cm^{-1} downshift (Figure 2B bottom).[14] This estimated shift results in a good simulation of the data. Thus, we conclude that the (+) 1687 and (−) 1675 cm^{-1} bands of D84 shift to (−) 1644 and (+) 1635 cm^{-1}, respectively (red dashed-shaded peaks in Figure 2B top). These frequencies and isotope shifts are typical of a ν_{as} band that originates from a unidentate carboxylate ligand to a metal ion.[11a] A frequency downshift would not be expected from the

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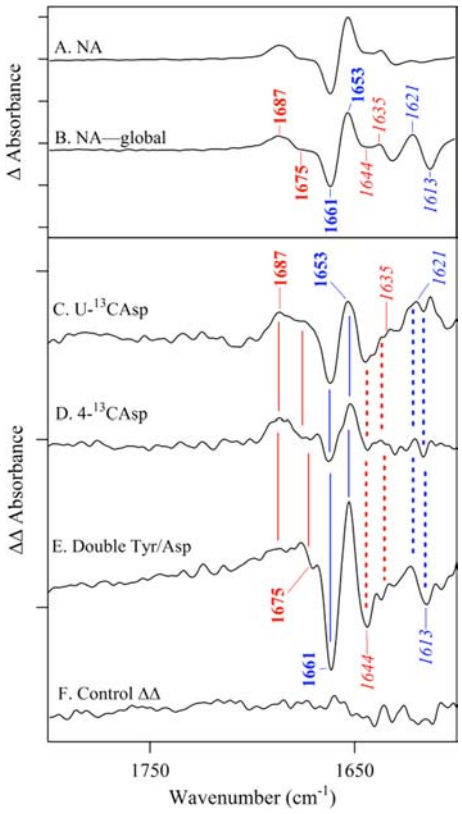

Figure 3. Reaction-induced FT-IR spectra associated with Y122O• reduction by HU, recorded at 20 °C (also see Figures S3 and S4). (A) NA difference spectrum for the HU reaction. (B–E) Isotope-edited HU spectra: (B) NA minus ^{13}C global (all C's); (C) NA minus [U-$^{13}C_4$]Asp (uniform); (D) NA minus [4-^{13}C]Asp (side chain); (E) NA minus [4-^{13}C]Asp/[1-^{13}C]Tyr (double label). (F) Control double-difference spectrum, providing an estimate of the baseline. The spectra are offset along the *y* axis for comparison. Reactions were performed in an FT-IR sample cell equipped with either (A,B) ~6 μm spacer or (C–F) 50 μm spacer. β2 concentrations were (A,B) 250 μM and (C–F) 100 μM in 5 mM HEPES, pD 7.6. The HU concentration was 50 mM in the same buffer. Red and blue labels represent assignments to ν_{as} and amide-I bands of D84, respectively (Table S1). Bands labeled in bold and italics represent NA and the ^{13}C isotopologue, respectively. Tick marks are 2.5 × 10^{-4} AU. (E) and (F) were baseline-corrected with a straight-line fit for presentation purposes.

change in iron oxidation state alone.[15] However, a decrease in H-bonding to a unidentate ligand (modeled as a carboxylate) weakens ν_{as}.[16] Thus, we attribute the spectral shift 1687→1675 cm^{-1} to the expected decrease in H-bonding to D84 when the metal–Y122O• cluster is reduced (Figure S1).[12,17] This experiment conclusively assigns the 1687 and 1675 cm^{-1} bands to D84 and establishes that D84 ν_{as} is a sensor for H-bonding changes in the diferric–Y122O• cofactor.

To test whether D84 H-bond changes are associated with Y122O• reduction alone, a comprehensive set of isotope-labeling experiments were conducted for the HU reaction (Figure 3). HU reduces only the tyrosyl radical and not the diferric cluster (see Figure S2A,B). The HU-mediated reaction-induced FT-IR spectrum of NA β2 is presented in Figure 3A. To construct the isotope-edited spectrum in Figure 3B, β2 was labeled at all C's (global, ~90%). These data demonstrate that the vibrational bands in Figure 3A reflect atomic displacements of β2 C-atoms.

To identify bands arising from vibrational displacements of Asp C-atoms, the [U-^{13}C]Asp (uniform) isotope-edited spectrum was acquired with HU (Figure 3C). This spectrum resembles the corresponding [U-^{13}C]Asp isotope-edited spectrum collected with HA (Figure 2B top). For example, the D84 ν_{as} bands at 1687 and 1675 cm^{-1} contribute to the HU spectrum (Figure 3C, red labels). Furthermore, isotope shifts from (+) 1687 to (−) 1644 cm^{-1} (Δ = −43 cm^{-1}) and (−) 1675 to (+) 1635 cm^{-1} (Δ = −40 cm^{-1}) (Table S1) were observed. These changes are congruent with the expected isotope shifts from the simulation in Figure 2B bottom.

In the [U-^{13}C]Asp spectrum, additional bands at (−) 1661 and (+) 1653 cm^{-1} (Figure 3C, blue labels) were isotope-shifted to (+) 1621 and (−) 1613 cm^{-1}, respectively (Table S1). These frequencies are typical of amide-I (C=O) vibrations.[18] These bands—also observed in the HA data—were assigned to the D84 amide-I band (Figure 2B top). To investigate the origin of these amide-I bands at 1661 and 1653 cm^{-1}, the side chain of Asp was specifically labeled (4-^{13}C, ~40%; Figure 3D). There was no significant isotope scrambling into the D84 amide bond in this isotopologue (see the SI). The persistence of amide-I (Figure 3D) and also amide-II (CN/NH) frequencies (Figure S4 and Table S1) in the isotope-edited spectrum suggests that the atomic motions of the D84 amide and side chain are coupled. We conclude that D84 contributes to the spectrum in the 1687/1675 (ν_{as}) and 1661/1653 cm^{-1} (amide-I) regions.

Redox changes at Y122O• may be associated with changes in amide vibrations of this Tyr. To show that those putative Tyr amide-I contributions can be distinguished from those of the Asp amide group, β2 was double-labeled at the Tyr amide bond ([1-^{13}C]Tyr) and the Asp side chain ([4-^{13}C]Asp) in the same β2 sample. The isotope-edited spectrum (Figure 3E) exhibited a significant intensity increase in the amide-I region relative to the results of Asp labeling alone (Figure 3C,D). This experiment provides further evidence that the 1661 and 1653 cm^{-1} bands in Figure 3C,D can be assigned to the D84 amide bond.

To summarize (Figure 4), isotope-edited and reaction-induced FT-IR spectra show that reduction of Y122O• by HU causes a change in the vibrational frequency of a unidentate metal ligand, D84. The observed 12 cm^{-1} downshift (1687→1675 cm^{-1}) for ν_{as} (Table S1) is consistent with a decrease in H-bond strength to D84 when the radical is reduced.[16] The frequencies and isotope shifts are similar to those observed when the diferric

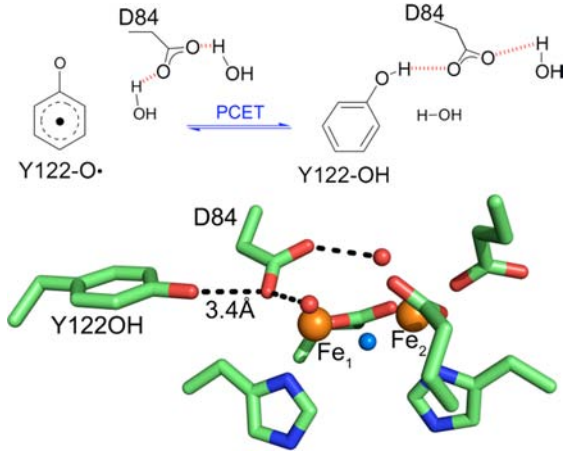

Figure 4. Top: Schematic of the D84 H-bond shift, which is linked to Y122O• reduction (PCET) by HU. Bottom: Structure of met (Fe^{3+}–Y122OH) β2. Waters and oxygen are shown as red and blue spheres, respectively.[12b,19] The Y122OH–D84 O–O distance is shown.

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cluster is reduced. While there are structures of the met (Fe^{3+}–Y122OH) state of the enzyme, there is no X-ray structure of the active (Fe^{3+}–Y122O$^{\bullet}$) form. However, EPR and vibrational spectroscopy provide evidence for a redox-linked conformational change between the Y122OH and Y122O$^{\bullet}$ states in isolated β2.[10,12b,20] In the high-resolution X-ray structure, reduced Y122OH is 3.4 Å from D84 (Figure 4). Changes in the backbone and ring dihedral angles deduced from UV Raman spectra[20] predict that oxidized Y122O$^{\bullet}$ is >3.4 Å from D84 and thus is not expected to be H-bonded to this carboxylate iron ligand. Previous magnetic resonance studies also concluded that Y122O$^{\bullet}$ is not H-bonded.[21] In the met form, D84 is predicted to H-bond to two water molecules bound to Fe_1 and Fe_2 (Figures 1, 4, and S1).[12] These water molecules are not present in the reduced protein (Figure S1) but are proposed to persist in the Fe^{3+}–Y122O$^{\bullet}$ state.[19]

Figure 4 diagrams a proposed redox-induced rotation of Y122O$^{\bullet}$ that translates the Tyr phenolic oxygen relative to Fe_1.[12b,20] This translation of the CO group can create a H-bond between Y122OH and D84, thereby weakening H-bonds between D84 and putative bound water molecules. Such a change in H-bonding to D84 is consistent with the D84 frequency shifts detected here. This H-bonding change can be driven by the expected pK_a change when Y122OH and Y122O$^{\bullet}$ are compared. While the pK_a of Y122OH is >9.6,[5] the pK_a of the Y122O$^{\bullet}$ radical is <0.[22]

An interaction between Y122OH redox reactions and H-bonding shifts in the iron cluster is supported by the literature. Quantum-mechanical calculations suggested that structural rearrangements around the diiron cluster may be necessary to promote PCET reactions.[19,23] Specifically, D84 was proposed in a preorganized proton-transfer pathway for cluster assembly and nucleoside diphosphate reduction.[19,23] The midpoint potential of the iron cluster was reported to be responsive to redox changes at Y122OH and changed from −115 to −163 mV when Y122OH was oxidized.[24] EPR spectroscopy of the mixed-valence (Fe^{2+}/Fe^{3+}; $S = {}^1/_2$) cluster suggested distinct conformations of iron ligands when the Y122O$^{\bullet}$ and Y122OH forms were compared.[25]

We show here that, when conducted with isotope labeling, reaction-induced FT-IR spectroscopy identifies protein dynamics in the form of carboxylate ligand reorganization. Our results are a proof of concept that single amino acid shifts in β2 can be detected. Also, this work shows that ν_{as} of iron-bound D84 is a marker for electrostatic changes in the metal center. We conclude that D84 H-bond shifts accompany PCET reactions at Y122O$^{\bullet}$ in the isolated β2 subunit. This is significant because even small shifts in H-bond distances can significantly alter PCET rates.[26] It is likely that the reactivity of diiron and other metal-containing proteins involves strict control over carboxylate H-bonding and ligation. Thus, our method provides an incisive approach to identify and describe the redox-linked carboxylate reorganizations that govern catalysis in metalloproteins.[1,27]

■ ASSOCIATED CONTENT

Supporting Information

Materials and methods, vibrational assignments and isotope shifts, structures, kinetic scans, and FT-IR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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