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Redox-Linked Changes to the Hydrogen Bonding Network of Ribonucleotide Reductase $\beta 2$

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Abstract

Ribonucleotide reductase (RNR) catalyzes the conversion of nucleoside diphosphates (NDPs) to 2'-deoxynucleotides, a critical step in DNA replication and repair in all organisms. The class Ia RNRs, which are found in aerobic bacteria and all eukaryotes, are a complex of two subunits: $\alpha 2$ and $\beta 2$. $\beta 2$ contains an essential diferric-tyrosyl radical (Y122O•) cofactor required to initiate reduction of NDPs in the $\alpha 2$ subunit. Here, we investigate the Y122O• reduction mechanism in *E. coli* $\beta 2$ by hydroxyurea (HU), a radical scavenger and cancer therapeutic agent. We test the hypothesis that Y122OH redox reactions cause structural changes at the diferric cluster. The reduction of Y122O• is studied using reaction-induced FT-IR spectroscopy and [¹³C]-aspartate labeled $\beta 2$. These spectra, Y122O•–Y122OH, provide evidence that the Y122OH redox reaction is associated with a frequency change to the asymmetric vibration (ν_{as}) of D84, a unidentate ligand to the diferric cluster. The results are consistent with a redox-induced shift in hydrogen bonding between Y122OH and D84, which may regulate proton transfer reactions on the HU-mediated inactivation pathway in isolated $\beta 2$.

Keywords

Carboxylate ligands; tyrosyl radical; diferric cluster; hydrogen bonding networks; vibrational spectroscopy

Reorganization of metal-bound carboxylates is proposed to play a significant role in modulating the reactivity of many non-heme diiron requiring proteins.^{1–2} 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 $\beta 2$ subunit of class Ia ribonucleotide reductase (RNR), which contains an essential tyrosyl radical (Y122O•)-diferric cofactor (Fig. 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 Fourier transform infrared (FT-IR) spectroscopy is unique among the biophysical methods for its ability to monitor subtle shifts to carboxylate ligands in real time. Here, we utilize the inherent sensitivity of FT-IR spectroscopy to detect

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Supporting Information. Materials, structures (Fig. S1), kinetic scans (Fig. S2), and FT-IR spectra (Figs. S3–S4). Figs. S5–S7 were used to estimate isotope incorporation. This material is available free of charge via the Internet at <http://pubs.acs.org>.

the reorganization of carboxylate D84 in the β_2 diferric-YO• cluster and thus showcase a powerful method to monitor these important changes.

RNR catalyzes the reduction of ribonucleotides to the corresponding deoxy form and, therefore, is a key participant in DNA biosynthesis and repair. It is composed of two subunits that form the active $(\alpha_2)_n\beta_2$ ($n = 1$ or 3) complex.^{3–4} In β_2 , there are three different scenarios in which carboxylate rearrangements/H bonding changes may occur (Fig. 1). Carboxylate shifts are known to accompany O₂-activation of the diferrous β_2 during assembly of the active Y122O•-diferric state (Fig. 1A). During turnover (Fig. 1B), Y122O•- β_2 generates a cysteine radical, C439•, at the substrate binding site in α_2 to initiate substrate reduction. This process involves reversible, proton-coupled electron transfer (PCET) over 35 Å between Y122O• and C439.⁵ Nucleotide reduction is limited by a conformational gate,⁶ which may be regulated by proton transfer to Y122O• from the metal cluster.⁷ Thus, changes in hydrogen bonding accompanying reversible Y122O• reduction are also predicted during catalysis. In this work, we show that the asymmetric stretching vibration (ν_{as}) of a carboxylate ligand, D84, is a sensor for hydrogen bonding changes in the diiron cluster.

To achieve this aim, we employed hydroxyurea (HU) to reduce Y122O• (Fig. 1C). HU was chosen to trigger the single-electron reduction of the diferric-Y122O• cofactor to the diferric-Y122OH or “met” state (Fig. S1).^{8–9} This process was monitored kinetically (Fig. S2) by loss in absorbance at 410 nm, associated with Y122O•. The rate constant for Y122O• reduction in H₂O was 0.33 M⁻¹s⁻¹, similar to previously reported values.¹⁰ The FT-IR experiments are conducted in D₂O buffers (see Supporting Information); therefore, HU kinetics were also evaluated under these conditions (Fig. S2A). Comparison of the second-order rate constants for Y122O• reduction obtained in 100% D₂O and 100% H₂O reveal a large solvent isotope effect, SIE = $k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}} = 15 \pm 1$ ($k_{\text{H}_2\text{O}} = 0.33 \text{ M}^{-1} \text{ s}^{-1}$; $k_{\text{D}_2\text{O}} = 0.023 \text{ M}^{-1} \text{ s}^{-1}$) at 20°C. Previous studies have shown that the Y122O• reaction with HU is non-saturable, suggesting that an initial, specific binding of HU to β_2 is not required for radical reduction.⁹ While the origin of the large SIE is not known, it could be consistent with conformational gating,^{7,11} simultaneous, multiple proton transfers, and/or proton tunneling.¹²

The reaction-induced FT-IR technique has been described previously.¹³ Vibrational spectra are recorded during the HU-mediated reduction of Y122O•, and the difference spectra are generated, representing Y122O•-minus-Y122OH (Figs. 2A and S3). Isotope-based assignments of FT-IR spectral features are possible through the construction of double difference spectra, called isotope-edited spectra (Fig. 2A). Difference FT-IR data collected independently from natural abundance (NA) and specific isotopically labeled (e.g. ¹³C) samples are subtracted (Figs. 2A and S4) to identify vibrational bands that are changed in amplitude and/or frequency by incorporation of the heavier isotope. Previously, we used this approach and [²H₄]-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.¹³

In addition to changes associated with Y122O• and Y122OH, the HU-derived difference spectra (Fig. 2A) will reflect other coupled changes in the environment of Y122OH, which may include structural changes at iron cluster ligands. D84 is a metal ligand, and thus its vibrational signature can be easily distinguished from that of free carboxylates or carboxylic acids.^{14–15}

As shown in Fig. 2A, bands in the 1680–1670 cm⁻¹ region were observed in the HU difference spectrum. This is a region that is expected to reflect frequency changes in unidentate carboxylate ligands.¹⁴ However, there is considerable variation in the expected

frequencies of carboxylate ligands, depending on metal oxidation state, ligand geometry, and hydrogen bonding.

To unambiguously identify the characteristic bands associated with hydrogen bond shifts at D84, we acquired the reaction-induced FT-IR spectrum associated with the reduction of Y122O• and the iron cluster. All available X-ray structures predict that D84 loses its hydrogen bonding to bound water molecules when the iron cluster is reduced (Fig. S1). This loss of hydrogen bonding and oxidation state change is expected to alter the vibrational frequencies of D84, the only aspartate ligand to the iron cluster.^{16–17}

To reduce both Y122O• and the iron cluster, hydroxylamine (HA) was employed. In H₂O, the reduction of the diferric cluster by HA proceeds at a much slower rate than Y122O•.¹⁸ However, in D₂O, the optical spectra (Fig. 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 iron cluster and Y122O• reduction.

To assign bands to D84 in the HA spectrum, an isotope-edited spectrum was created, through the use of a U-¹³C₄Asp isotopologue of β2 (Fig. 2B, top). The U-¹³C₄Asp sample was produced in an Asp auxotroph and was ~40% labeled (see Supporting Information). The isotope-edited, reaction-induced FT-IR spectrum, which identifies all Asp vibrational modes involving carbon displacement, is shown in Fig. 2B, top. In the region presented, the expected contributions arise from normal modes ν_{as} of the Asp side chain and amide I (peptide C=O) of the Asp amide bond. In Fig. 2B, top, bands of NA Asp will be positive in the Y122O• state and negative in the Y122OH state. Isotope-shifted bands appear with opposite sign.

In the HA isotope-edited spectrum (Fig. 2B, top, red solid fill), two bands were observed at (+) 1687 and (–) 1675 cm⁻¹. Because these bands are observed in the isotope-edited spectrum, these spectral features are assignable to D84. To predict the ¹³C isotope shifts for these bands, a simulated isotope-edited spectrum (Fig. 2B, bottom) was generated assuming the expected 43 cm⁻¹ downshift.¹⁹ This estimated shift results in a good simulation of the data (Fig. 2B, bottom). Therefore, we conclude that the (+) 1687/(–) 1675 bands of D84 shift to (–) 1644/(+) 1635 cm⁻¹ (Fig. 2B, top, red shaded fill). These frequencies and isotope shifts are typical of a ν_{as} that originates from a unidentate carboxylate ligand to a metal ion.¹⁴ A frequency downshift is not expected from the iron oxidation state change alone.²⁰ However, a decrease in hydrogen bonding to a unidentate ligand (modeled as a carboxylate) weakens ν_{as}.²¹ Therefore, we attribute the spectral shift observed, 1687 → 1675 cm⁻¹, to the expected decrease in hydrogen bonding to D84 when the Y122O•-metal cluster is reduced (Fig. S1).^{16–17,22} This experiment conclusively assigns the 1687/1675 cm⁻¹ bands to D84 and establishes that D84 ν_{as} is a sensor for hydrogen bonding changes at the Y122O•-diferric cofactor.

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

To identify bands arising from vibrational displacements of Asp carbon atoms, the U-¹³CAsp (uniform) isotope-edited spectrum was acquired with HU (Fig. 3C). This

spectrum resembles the corresponding U-¹³CAsp isotope-edited spectrum, collected with HA (Fig. 2B, top). For example, D84 ν_{as} bands at 1687 and 1675 cm⁻¹ contribute to the HU spectrum (Fig. 3C, red labels). Further, isotope shifts from (+) 1687 to (-) 1644 ($\Delta = -43$) cm⁻¹ and (-) 1675 to (+) 1635 ($\Delta = -40$) cm⁻¹ (Table S1) were observed. These changes are congruent with the expected isotope shifts from the simulation in Fig. 2B, bottom.

In the U-¹³CAsp spectrum, additional bands, at (-) 1661/(+) 1653 cm⁻¹, were observed (Fig. 3C, blue labels) and were isotope-shifted (Table S1) to (+) 1621/(-) 1613 cm⁻¹. These frequencies are typical of amide I (C=O) vibrations.²³ These bands were also observed in the HA data and were assigned to the D84 amide I band (Fig. 2B, top). To investigate the origin of these amide I 1661/1653 cm⁻¹ bands, the side chain of Asp was specifically labeled (4-¹³C, ~40%; Fig. 3D). There is no significant isotope scrambling into the D84 amide bond in this isotopologue (see Supporting Information). Therefore, the persistence of amide I (Fig. 3D) and, also, amide II (CN/NH) frequencies (Fig. 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 cm⁻¹ region (ν_{as}) and in the 1661/1653 cm⁻¹ region (amide I).

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, $\beta 2$ was double-labeled at the Tyr amide bond (1-¹³C Tyr) and the Asp side chain (4-¹³C Asp) in the same $\beta 2$ sample. The isotope-edited spectrum (Fig. 3E) exhibited a significant intensity increase in the amide I region, compared to the results of Asp labeling alone (Figs. 3C and D). This experiment provides further evidence that the 1661/1653 cm⁻¹ bands in Figs. 3C and D can be assigned to the D84 amide bond.

To summarize (Fig. 4), isotope-editing 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 \rightarrow 1675 cm⁻¹) for ν_{as} (Table S1) is consistent with a decrease in hydrogen bond strength to D84 when the radical is reduced.²¹ The frequencies and isotope shifts are similar to those observed when the diferric cluster is reduced. While there are structures of the met (Y122OH, Fe³⁺) state of the enzyme, there is no X-ray structure of the active (Y122O•, Fe³⁺) form. However, EPR and vibrational spectroscopy have provided evidence for a redox-linked conformational change between Y122OH/Y122O• states in isolated $\beta 2$.^{13,17,25} In the high-resolution X-ray structure, reduced Y122OH is 3.4 Å from D84 (Fig. 4). Backbone and ring dihedral angle changes, deduced from UV Raman spectra,²⁵ predict that oxidized Y122O• is >3.4 Å from D84 and is, thus, not expected to be hydrogen bonded to this carboxylate iron ligand. Previous magnetic resonance studies have also concluded that Y122O• is not hydrogen bonded.²⁶ In the met form, D84 is predicted to hydrogen bond to two water molecules, which are bound to Fe₁ and Fe₂ (Figs. 1, 4, and S1).¹⁶⁻¹⁷ These water molecules are not present in the reduced protein (Fig. S1), but are proposed to persist in the Y122O•-Fe³⁺ state.²⁴

Fig. 4 diagrams a proposed redox-induced rotation of Y122O•, which translates the Tyr phenolic oxygen relative to Fe₁.^{17,25} This translation of the CO group can create a hydrogen bond between Y122OH and D84 and thereby weaken a hydrogen bond between D84 and putative bound water molecules. Such a hydrogen bonding change to D84 is consistent with the D84 frequency shifts detected here. This hydrogen-bonding change can be driven by the expected pK_a change, when Y122OH and Y122O• radical are compared. While the pK_a of Y122OH is greater than 9.6,⁷ the pK_a of the Y122O• radical is less than 0.²⁷⁻²⁸

An interaction between Y122OH redox reactions and hydrogen bonding shifts at the iron cluster is supported by the literature. Quantum mechanical calculations have proposed that structural rearrangements around the diiron cluster may be necessary to promote PCET reactions.^{24,29} Specifically, D84 has been proposed in a pre-organized proton transfer pathway for cluster assembly and NDP reduction.^{24,29} 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.³⁰ EPR spectroscopy of the mixed-valence ($\text{Fe}^{2+}\text{Fe}^{3+}$; $S=1/2$) cluster suggested distinct conformations of iron ligands when the Y122O• and Y122OH forms were compared.³¹

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 can be detected in $\beta 2$. 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 hydrogen bond shifts accompany PCET reactions at Y122O• in the isolated $\beta 2$ subunit. This is significant, because even small shifts in hydrogen bond distances can significantly alter PCET rates.³² It is likely that the reactivity of diiron and other metal-containing proteins involves strict control over carboxylate hydrogen bonding and ligation. Therefore, our method provides an incisive approach to identify and describe the redox-linked carboxylate reorganizations that govern catalysis in metalloproteins.^{1-2,33}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

RNR	ribonucleotide reductase
FT-IR	Fourier transform infrared
HA	hydroxylamine
HU	hydroxyurea
PCET	proton-coupled electron transfer

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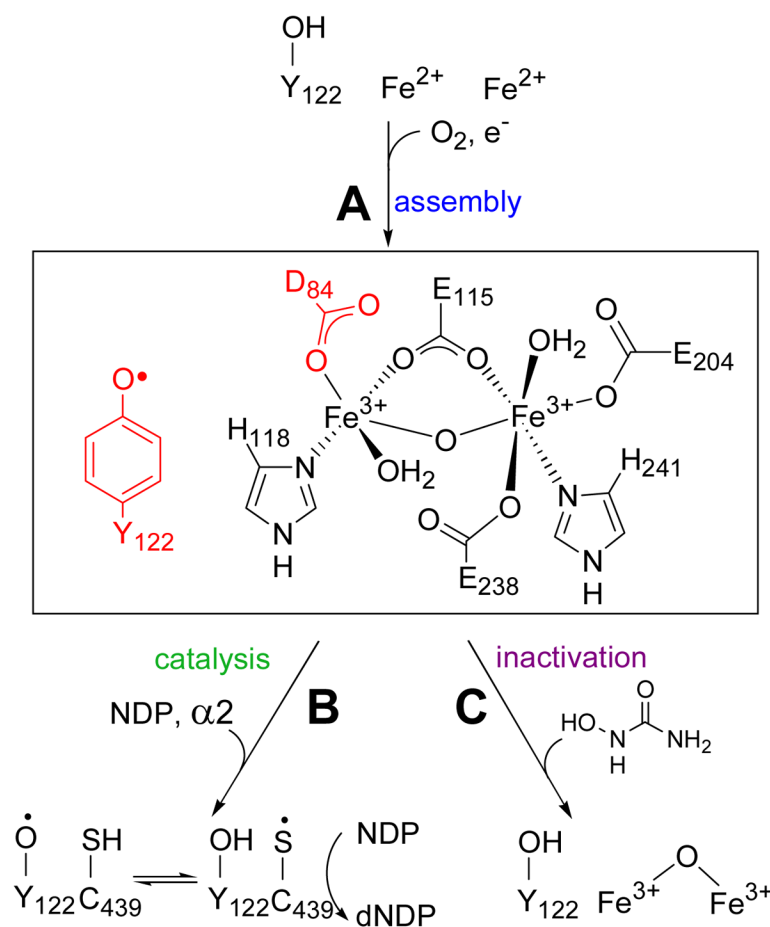


Figure 1. Possible scenarios for carboxylate reorganization at the diiron cofactor in *E. coli* class Ia $\beta 2$. The active cofactor is first assembled by reaction of diferrous $\beta 2$, O_2 , and reductant (A). The stable $\text{Y122O}\cdot$ is reduced concomitantly with C439 oxidation by reversible, long-range PCET during catalysis (B). Alternatively, the cofactor may be rendered inactive by small molecule-mediated reduction of $\text{Y122O}\cdot$ (C). In this report, we monitor changes to the amino acids in red with HU-mediated inactivation.

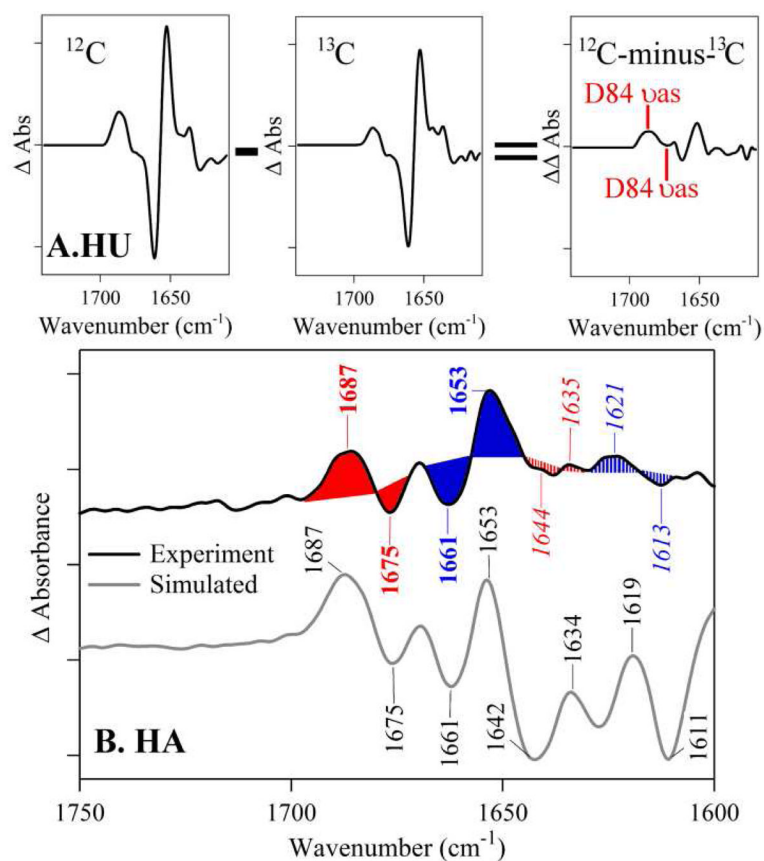


Figure 2. Schematic of the FT-IR isotope-edited spectrum, reflecting [4- ^{13}C]-Asp (40%) labeling and reduction of Y122O \bullet with HU (A). Band assignments to ν_{as} of D84 are labeled in red. (B) shows the NA-minus-U- $^{13}\text{C}_4$ Asp isotope-edited spectrum (top, see also Figs. S3–S4) for the HA reaction. The reaction was 100 μM β_2 and 25 mM HA in 5mM Hepes, pD 7.6 (20 $^\circ\text{C}$, 50 μm spacer). Band assignments were established by spectral simulation (bottom). The bottom trace is the simulated isotope-edited spectrum, which accounts for the data and was produced from the NA spectrum in Fig. S3F assuming ^{13}C shifts of -43 cm^{-1} . Red and blue labels represent assignments to ν_{as} and amide I of D84, respectively. Bands labeled in bold face (solid shading) and italics (dashed shading) represent NA and ^{13}C isotope, respectively. Tick marks in (B) are 2×10^{-4} AU.

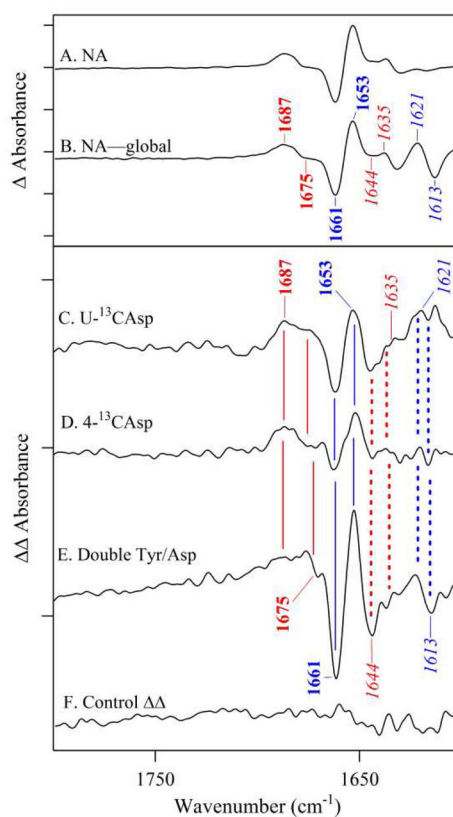
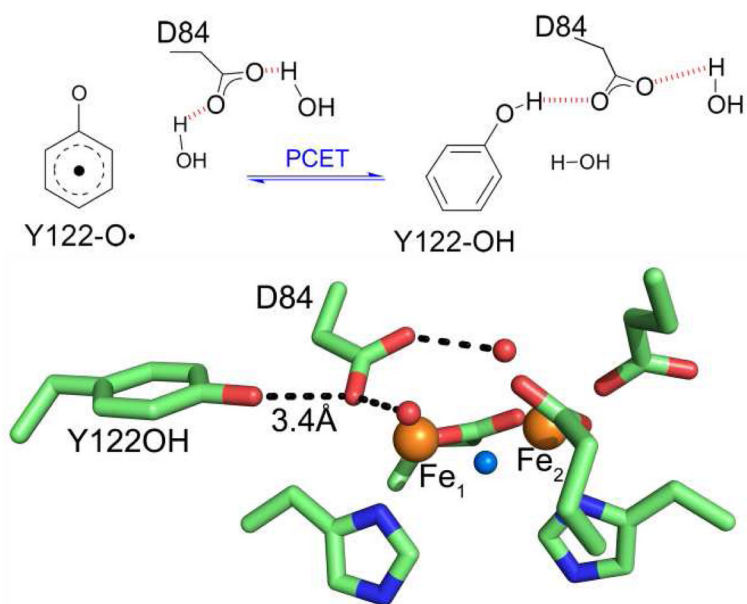


Figure 3.

Reaction-induced FT-IR spectra associated with Y122O• reduction by HU, recorded at 20°C (see also Figs. S3 and S4). The NA difference spectrum for the HU reaction is shown in (A). In (B–E), isotope-edited HU spectra were constructed, (B) NA–minus– ^{13}C global (all carbons), (C) NA–minus–U- $^{13}\text{C}_4$ Asp (uniform), (D) NA–minus–4- ^{13}C Asp (side chain), and (E) NA–minus–4- ^{13}C Asp/1- ^{13}C Tyr (double label). The spectra were offset along the y-axis for comparison. Reactions were collected in a FT-IR sample cell equipped either with a (A–B) ~ 6 μm or a (C–F) 50 μm spacer. The $\beta 2$ 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 of D84, respectively (Table S1). Bands labeled in bold face and italics represent NA and ^{13}C isotopologue, respectively. Tick marks are 2.5×10^{-4} AU. (F) shows a control double difference spectrum, which provides an estimate of the baseline. (E) and (F) were baseline corrected with a straight-line fit for presentation purposes.

**Figure 4.**

Top: Schematic of D84 hydrogen bond shift, which is linked to Y122O• reduction (PCET) by HU. **Bottom:** Structure of met (Y122OH, Fe³⁺) β₂. Waters and oxygen are red and blue spheres, respectively.^{17,24} Y122OH-D84 O-O distance is shown.