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Redox-Linked Structural Changes in Ribonucleotide Reductase

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Ribonucleotide reductase (RNR) catalyzes the reduction of ribonucleotides to deoxyribonucleotides.[1−3] Class I RNRs are composed of a 1:1 complex of two homodimeric proteins, α2 and β2. α2 contains the binding site for substrates and allosteric effectors that govern turnover rate and specificity, and β2 houses the essential diferric-tyrosyl radical (residue 122, Y122•) cofactor.[1−4] Y122• acts as a radical initiator, generating a thiyl radical in α2[5], ∼35 Å removed.[6−8] Long distance proton coupled electron transfer (PCET) is facilitated by a series of amino acid radical intermediates, which serve to accelerate the reaction rate into a physiologically relevant range.[9−15]

In this work, vibrational spectroscopy is used to show that electron transfer to and from the tyrosyl radical (Y122•) in RNR is coupled to a conformational change in the β2 subunit. Such redox-linked conformational changes are important because they can modulate the interaction of Y122 with its hydrogen bonding partner and provide local, structural control of its PCET reactions.

In previous work, high resolution magnetic resonance studies of Y122•[16] were performed. Comparison with the X-ray structure of reduced β2 suggested that a $C_\alpha-C_\beta$ single bond rotation may occur when Y122 is oxidized. This single bond rotation was attributed to an electrostatic repulsion between Asp-84 and Y122• and would disconnect Y122• from the hydrogen bond network at the diiron site. A Y• conformational rearrangement has also been proposed to occur with Y oxidation in pentapeptides and tyrosinate at 85 K.[17]

To identify redox-linked structural changes associated with electron transfer reactions in β2, we have performed FT-IR spectroscopy on the purified *E. coli* subunit in ^2H_2O buffer (Supporting Information, Figures S1 and S2). FT-IR spectroscopy has emerged as a powerful tool in elucidating enzyme mechanisms (reviewed in ref 18). This approach has been used to study redox-active tyrosines in other proteins, such as photosystem II[19] and cytochrome *c* oxidase.[20]

The reaction-induced difference spectrum, associated with the reduction of Y122•, is presented in Figures 1A and 2A. These data were acquired by one-electron reduction of Y122• with hydroxyurea[21−24] over 10 min. Based on the rate constant in ^2H_2O (Figures S3 and S4), an estimated ∼60% of Y122• was reduced during the 10 min FT-IR measurement.

In the difference spectrum shown in Figure 1A, unique bands of the tyrosyl radical, Y122•, will be positive features; unique bands of Y122 will be negative features. Using model compounds and DFT calculations, it has been shown that oxidation of tyrosine leads to an upshift of the CO stretching vibration to 1516 cm^{-1} (υ7a) and to perturbations of the aromatic ring stretching frequencies, at ∼1600 (υ8a) and ∼1500 (υ19a) cm^{-1} (Figures 1A and 2A; see ref 25 and references therein). Oxidation of the tyrosine aromatic side chain in peptides and in tyrosinate at pH 11 gave similar results for the CO and υ19a ring stretching modes (Figure 1D and E; refs

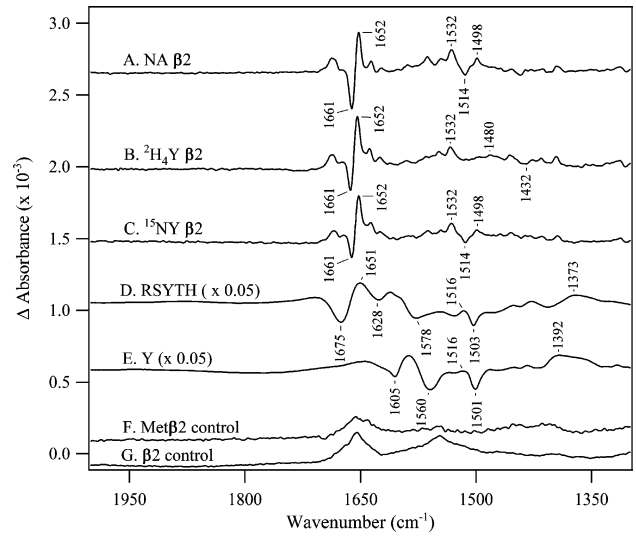

Figure 1. Reaction-induced FT-IR spectra, monitoring redox-linked structural changes induced by Y122• reduction with 50 mM hydroxyurea. Spectra (A−C, F, and G) were acquired at 20 °C in ^2H_2O buffer. In (A), the (250 μM) β2 sample was natural abundance, in (B) $^2H_4$-tyrosine labeled, and in (C) ^{15}N-tyrosine labeled. In (D) and ((E), Y• was generated using 266 nm photolysis at 80 K in the RSTYH peptide (D) or in tyrosinate (E) at p^{2}H 11. (F) is a (250 μM) Metβ2 control difference spectrum, which lacks Y122•, but was mixed with hydroxyurea. (G) is a (250 μM) β2 control difference spectrum, in which β2 was mixed with buffer, instead of hydroxyurea. The difference spectra were constructed: Y•-minus-Y. The spectra are averages from 8 (A), 6 (B), 8 (C), 5 (D), 3 (E), 6 (F), and 11 (G) samples.

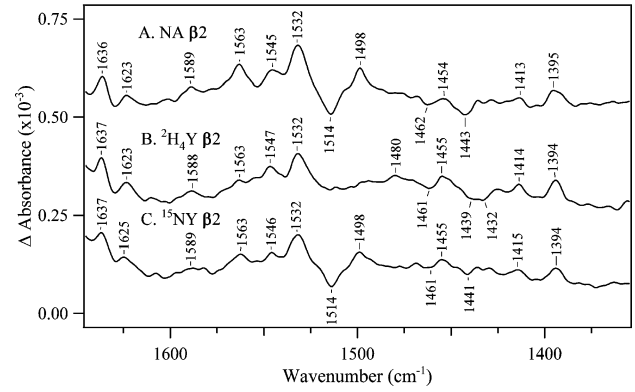

Figure 2. 1700−1300 cm^{-1} region of the reaction-induced FT-IR spectrum, monitoring redox-linked structural changes induced by Y122• reduction with 50 mM hydroxyurea. Spectra A−C are expanded and repeated from Figure 1A−C, respectively.

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17, 26). Previous Raman studies of the *E. coli* and mouse $\beta 2$ subunits have attributed bands at 1498[27] and 1515[28] cm^{-1}, respectively, to the CO vibration.

To identify Y122•/Y122 contributions to the natural abundance spectrum (Figures 1A and 2A), the $\beta 2$ subunit was labeled with the $^2H_4$-tyrosine isotopomer (Figures 1B and 2B). $^2H_4$ tyrosine labeling resulted in a downshift of a negative band at 1514 cm^{-1} to 1432 cm^{-1} (Figures 2A and B). This band was not observed in a negative Met$\beta 2$ control (Figure 1F) or in a negative control, in which the $\beta 2$ subunit was mixed with buffer instead of hydroxyurea (Figure 1G). Based on previous model compound studies and DFT calculations,[25] we assign this band to a ring stretching vibration (υ19a) of Y122. From DFT calculations, the expected isotope-induced downshift is 80 cm^{-1}.[25]

$^2H_4$ labeling also resulted in a downshift of a positive band at 1498 to 1480 cm^{-1} (Figures 2A and B). This band was not observed in the negative controls (Figure 1F or G) and is assigned to the CO stretching vibration (υ7a) of Y122•. From DFT calculations, the expected isotope-induced downshift is $\sim$20 cm^{-1}.[25] The assignment of the 1498 cm^{-1} band is in agreement with previous Raman studies.[27]

These data establish that the CO vibrational band of Y122• is downshifted $\sim$20 cm^{-1} from its frequency in Y• model compounds,[17,25] where it is observed as a positive band at 1516 cm^{-1} (Figure 1D and E). The symmetric ring stretching mode, υ8a, at negative 1605 cm^{-1} (Figure 1E) was not observed when Y• is generated in the pentapeptide (Figure 1D) or in $\beta 2$ (Figure 1A).

In addition to bands associated with tyrosine oxidation, the reaction-induced spectrum in Figure 1A reveals coupled, oxidation-induced effects on the protein environment. For example, Figure 1A exhibits a negative band at 1661 cm^{-1} and a positive band at 1652 cm^{-1}. This is a spectral region characteristic of amide I C═O vibrational bands (Figure S5).[29] These data suggest that Y122• reduction is coupled with a structural change in the $\beta 2$ subunit.

As a first step in interpretation of the amide I spectral contributions, we studied a pentapeptide, RSYTH, matching the sequence containing Y122 in the $\beta 2$ subunit.[17] The pentapeptide spectrum in ^2H_2O buffer (Figure 1D) exhibited several bands in the amide I region, at 1675 and 1651 cm^{-1}, which were not observed when the tyrosyl radical was generated in a powder tyrosinate sample (Figure 1E).[17] This result suggests that electron transfer reactions are linked with a structural change in one or more peptide bonds in the pentapaptide and in the $\beta 2$ sample. Because the pentapeptide is expected to have no defined structure in solution, the primary sequence must mediate this redox-linked interaction. We propose that this conformational change occurs at adjacent peptide bonds to Y122•.

^{15}N-labeling of tyrosine in $\beta 2$ was employed (Figure 1C and 2C) to test if the structural perturbation occurs at the S121−Y122 amide bond. The effect of ^{15}N labeling is expected to be detectable {for example, see ref 30}, because the amide I normal mode involves nitrogen displacement.[29] However, ^{15}N labeling had no significant effect on the reaction-induced spectrum (Figure 1A and C). This result is consistent with the conclusion that the redox-linked structural change does not perturb the Ser-Tyr amide linkage, but other amide bonds near Y122.

We hypothesize that the structural perturbation of the amide bond in the $\beta 2$ and the pentapeptide samples is driven by an electrostatic change. Electrostatic maps predict a change in aromatic ring charge distribution.[31] An oxidation-induced change in charge distribution will lead to alterations in the C−N bond ionic character, planarity, and force constant through a Stark effect. This interpretation is congruent with recent ESEEM studies of pentapeptide samples, which have shown sequence-dependent changes in nuclear quadrupole interactions in tyrosyl radicals.[32]

Redox-linked structural changes at Y122/Y122• may partially control PCET reactions during the RNR reaction cycle.[5,16] These conformational alterations are likely to cause changes in Y122 interactions with its hydrogen bonding partner.[16] Therefore, these structural changes may play a role in regulation of the Y122 midpoint potential and its reversible protonation/deprotonation reactions. This work provides spectroscopic evidence for dynamic structural changes in RNR.

Supporting Information Available: Description of the expression/purification of the $\beta 2$ subunit, the FT-IR methods, the kinetic studies, and acknowledgments. This information is available free of charge via the Internet at http://pubs.acs.org/.

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