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Redox-linked Structural Changes in Ribonucleotide Reductase

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Abstract

Ribonucleotide reductase (RNR) catalyzes the reduction of ribonucleotides to deoxyribonucleotides. Class I RNRs are composed of two homodimeric proteins, α2 and β2. The class Ia E. coli β2 contains dinuclear, antiferromagnetically coupled iron centers and one tyrosyl free radical, Y122•/β2. Y122• acts as a radical initiator in catalysis. Redox-linked conformational changes may accompany Y122 oxidation and provide local control of proton-coupled electron transfer reactions. To test for such redox-linked structural changes, FT-IR spectroscopy was employed in this work. Reaction-induced difference spectra, associated with the reduction of Y122• by hydroxyurea, were acquired from natural abundance, ²H⁴ tyrosine, and ¹⁵N tyrosine labeled β2 samples. Isotopic labeling led to the assignment of a 1514 cm⁻¹ band to the υ₁₉a ring stretching vibration of Y122 and of a 1498 cm⁻¹ band to the υ₇a CO stretching vibration of Y122•. The reaction-induced spectra also exhibited amide I bands, at 1661 and 1652 cm⁻¹. A similar set of amide I bands, with frequencies of 1675 and 1651 cm⁻¹, was observed when Y• was generated by photolysis in a pentapeptide, which matched the primary sequence surrounding Y122. This result suggests that reduction of Y122• is linked with structural changes at nearby amide bonds and that this perturbation is mediated by the primary sequence. To explain these data, we propose that a structural perturbation of the amide bond is driven by redox-linked electrostatic changes in the tyrosyl radical aromatic ring.

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 diferricytosyl radical (residue 122, Y122•) cofactor.¹⁻⁴ Y122• acts as a radical initiator, generating a thyl radical in α25, ~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.

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Supporting Information Available: Description of the expression/purification of the β2 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/.
In previous work, high resolution magnetic resonance studies of Y122• were performed. Comparison with the X-ray structure of reduced β2 suggested that a Cα-Cβ 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.

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 $^2$H$_2$O buffer (Supplemental information, Figs. 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 and cytochrome c oxidase.

The reaction-induced difference spectrum, associated with the reduction of Y122•, is presented in Figs. 1A and 2A. These data were acquired by one electron reduction of Y122• with hydroxyurea over 10 min. Based on the rate constant in $^2$H$_2$O (Figs. S3 and S4), an estimated ~60% of Y122• is reduced during the 10 min FT-IR measurement.

In the difference spectrum shown in Fig. 1A, unique bands of 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$^{-1}$ (Figs. 1A and 2A and see ref 25 and refs 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 (Fig. 1D and E and refs 17, 26). Previous Raman studies of the E. coli and mouse β2 subunits have attributed bands at 1498 and 1515 cm$^{-1}$, respectively, to the CO vibration.

To identify Y122•/Y122 contributions to the natural abundance spectrum (Figs. 1A and 2A), the β2 subunit was labeled with the $^2$H$_4$-tyrosine isotopomer (Figs. 1B and 2B). $^2$H$_4$ tyrosine labeling resulted in a downshift of a negative band at 1514 cm$^{-1}$ (υ7a) and to perturbations of the aromatic ring stretching frequencies, at ~1600 (υ8a) and ~1500 (υ19a) cm$^{-1}$ (Figs. 1A and 2A and see ref 25). 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 (Fig. 1D and E and refs 17, 26). Previous Raman studies of the E. coli and mouse β2 subunits have attributed bands at 1498 and 1515 cm$^{-1}$, respectively, to the CO vibration.

$^2$H$_4$ labeling also resulted in a downshift of a positive band at 1498 cm$^{-1}$ to 1480 cm$^{-1}$ (Figs. 2A and B). This band was not observed in the negative controls (Figs. 1F or G) and is assigned to the CO stretching vibration (υ7a) of Y122•. From DFT calculations, the expected isotope-induced downshift is 80 cm$^{-1}$.25

These data establish that the CO vibrational band of Y122• is downshifted ~20 cm$^{-1}$ from its frequency in Y• model compounds,17,25 where it is observed as a positive band at 1516 cm$^{-1}$ (Fig. 1D and E). The symmetric ring stretching mode, υ8a, at negative 1605 cm$^{-1}$ (Fig. 1E) was not observed when Y• is generated in the pentapeptide (Fig. 1D) or in β2 (Fig. 1A).

In addition to bands associated with tyrosine oxidation, the reaction-induced spectrum in Fig. 1A reveals coupled, oxidation-induced effects on the protein environment. For example, Fig. 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.\textsuperscript{29} These data suggest that Y122• reduction is coupled with a structural change in the β2 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 β2 subunit.\textsuperscript{17} The pentapeptide spectrum in \textsuperscript{2}H\textsubscript{2}O buffer (Fig. 1D) exhibited several bands in the amide I region, at 1675 and 1651 cm\textsuperscript{-1}, which were not observed when tyrosyl radical was generated in a powder tyrosinate sample (Fig. 1E).\textsuperscript{17} This result suggests that electron transfer reactions are linked with a structural change in one or more peptide bonds in the pentapeptide and in the β2 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•.

\textsuperscript{15}N-labeling of tyrosine in β2 was employed (Fig. 1C and 2C) to test if the structural perturbation occurs at the S121-Y122 amide bond. The effect of \textsuperscript{15}N labeling is expected to be detectable \{for example, see ref 30 \}, because the amide I normal mode involves nitrogen displacement.\textsuperscript{29} However, \textsuperscript{15}N labeling had no significant effect on the reaction-induced spectrum (Figs. 2A 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 β2 and the pentapeptide samples is driven by an electrostatic change. Electrostatic maps predict a change in aromatic ring charge distribution.\textsuperscript{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.\textsuperscript{32}

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

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**References**

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 2H2O buffer. In (A), the (250 μM) β2 sample was natural abundance, in (B) 2H4-tyrosine labeled, and in (C) 15N-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 pH 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.
The 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 2A, B and C are expanded and repeated from Fig. 1A, B and C, respectively.