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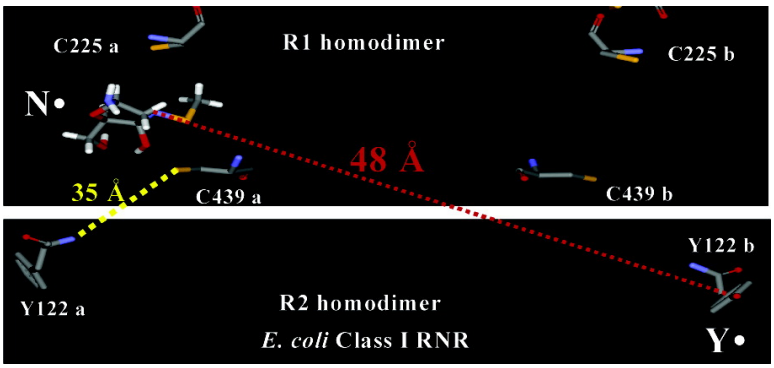

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EPR Distance Measurements Support a Model for Long-Range Radical Initiation in *E. coli* Ribonucleotide Reductase

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Escherichia coli ribonucleotide reductase (RNR) catalyzes the conversion of nucleoside diphosphates (NDPs) to deoxynucleoside diphosphates. The active protein is composed of two homodimeric subunits (R1 and R2) thought to form a 1:1 complex.[1] R1 binds the NDP substrates, and houses the essential cysteines (C225, C462, and C439) required for catalysis and the binding sites for the allosteric effectors that govern substrate specificity and turnover rates. R2 harbors the essential diiron tyrosyl radical cofactor on residue 122 (Y•). The chemistry of nucleotide reduction is moderately well understood,[2] and structures of R1[3] and of R2 are available.[4,5] A major unresolved issue, however, is the mechanism of radical initiation:[6] how the tyrosyl radical (Y•) in R2 generates a transient thiyl radical (C439•) in R1 required for nucleotide reduction. The current model for the radical initiation process involves a specific pathway composed of aromatic amino acids and traverses a distance of 35 Å.[3] The distance is derived from a docking model of the R1 and R2 structures and conservation of amino acids in the pathway. Y356 has recently been demonstrated to be one of the residues in the pathway.[7] It resides in the unstructured C-terminus of R2, and thus a substantial part of the electron-transfer pathway is not apparent from the available structural information. A method to measure the distance between Y122 and C439 in solution is imperative to the radical initiation model.[6] Using *p*ulsed *el*ectron-electron *do*uble *r*esonance (PELDOR) spectroscopy[8] and *d*ouble *q*uantum *c*oherence (DQC)[9] methods, we now report the first measurement of the distance between Y• in R2 and a nitrogen-centered radical (N•), attached to C225 in the active site of R1 (Figure 1b).

To generate the second paramagnetic species (N•, Figure 1b or c) required for the distance measurements by these methods, we have taken advantage of previous studies that have shown that *E. coli* RNR is rapidly inactivated by 2′-azido-2′-deoxyuridine-5′-diphosphate (N_3UDP).[11,12] Inactivation results from loss of the essential Y• on R2 and formation of a new, long-lived N• in the active site of R1. Three different paramagnetic pairs are theoretically possible from this experiment: Y•/Y•, N•/Y•, and N•/N• (Figure 1). A distinction between the Y•/N• and N•/N• is possible based on the enhanced relaxation properties of Y• adjacent to the diiron site relative to the N• located in R1 distant from the iron center.[13]

Incubation of *E. coli* RNR with N_3UDP and freeze quenching the reaction between 2 and 5 min[14] resulted in an EPR spectrum that contained 57% N• and 43% Y• (Figure 2A). The four-pulse standard and variable-time DEER (*d*ouble *e*lectron−*e*lectron *r*esonance) sequences[15,16] were applied at X-band in order to detect the distance between N• and Y• (Figure 1b). Figure 2B displays the echo modulation traces recorded at position 1 in the EPR line (Figure 2A), with pumping at the maximum of the Y• absorption

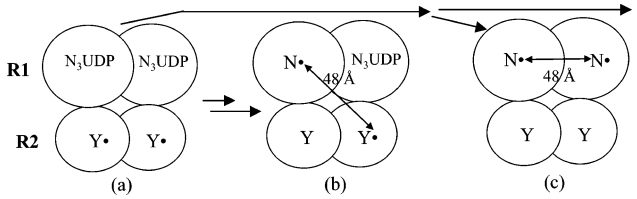

Figure 1. Possible models for radical distribution when R1R2 is inactivated by N_3UDP. (a) N_3UDP binds to each protomer of R1, which is in complex with R2. The radical transfer pathway is proposed to involve Y122, W48, and Y356 in R2 and Y731, Y730, and C439 in R1. (b) In one model, the Y• on one protomer of R2 is reduced concomitant with generation of N• from N_3UDP on the symmetry-related protomer of R1. (c) In the second model, the Y•s on each protomer of R2 are reduced concomitant with formation of N• on each protomer of R1. R2 contains ≈1.2 of 2 possible Y•s. Our recent PELDOR experiments[10] indicate that at least 25% of the Y•s in (a) are paired.

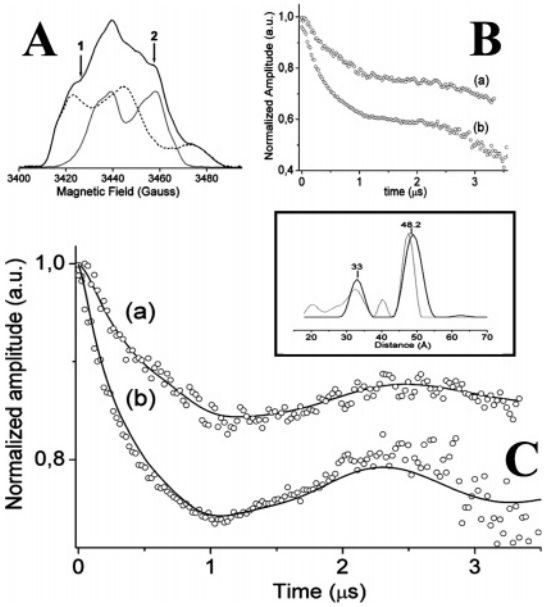

Figure 2. (A) Spin−echo detected spectrum (solid dark line) of the reaction mixture formed during the inhibition of *E. coli* RNR with N_3UDP: gray line, contribution from the Y•; dotted line, contribution from the N•. The arrows indicate the selected positions of pumping (2) and detection (1). (B) Normalized standard four-pulse DEER at 4 K (a) and variable-time DEER at 20 K (b). Pulses on the detection frequency ($\pi/2,\pi$) were 32 ns; t_{pump} = 20 ns. (C) Time traces after subtraction of a monoexponential decay. Solid lines: fit using distance-domain Tikhonov regularization. Inset: resulting distance distributions from trace a (solid line) and trace b (dotted line).

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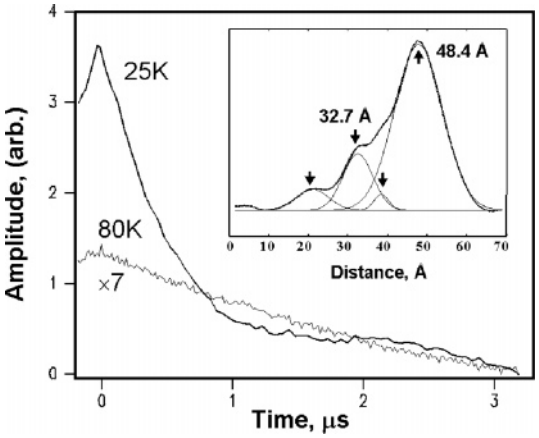

Figure 3. DQC signal of the reaction mixture of RNR with N_3UDP at 17.4 GHz; 3.2 and 6.2 ns $\pi/2$ and π pulses were employed (B_1 = ca. 30 G). DQC evolution time was 200 ns at 80 K versus 64 ns at 25 K. The pulse excitation was at the center of the Y• EPR spectrum. The inset shows the distribution in distances, $P(r)$, from the Tikhonov regularization. The $P(r)$ may be represented as a sum of four Gaussians centered at different distances, of which two distances can be assigned.

at position 2. These time traces show a clear oscillation superimposed on the echo decay. To determine the N•−Y• distance, the data were analyzed with the software DEERAnalysis 2004.[17] After subtraction of a monoexponential decay, fitting using the distance-domain Tikhonov regularization procedure gave the distance distributions shown in the inset of Figure 2. In both analyses, a dominant peak is observed at a mean value of 48.2 Å, and an additional minor contribution is observed at 33 Å (Y•−Y• pairs).[18]

In a second experiment with N_3UDP,[19] a six-pulse DQC sequence[9] was employed at 17.4 GHz, at 25 and 80 K (Figure 3). The oscillating signal at 25 K shows the presence of interacting paramagnetic species at well-defined distances. The distance analysis of the DQC data (Figure 3 inset) by Tikhonov regularization[20] confirms the X-band data, indicating the presence of radical pairs with average distances of ca. 33 Å (Y•−Y•) and 48.4 Å.[21] Given the signal-to-noise, the average distances are accurate to ±1 Å. In contrast, the DQC signal at 80 K (Figure 3) is typical of one originating from the intermolecular dipolar couplings in a uniformly dilute sample.[22] At 80 K, the only source of the DQC signal is associated with N•−N• pairs as the Y• is removed from the picture due its short T_2. The DQC evolution time was purposely increased to 200 ns at 80 K to guarantee removal of the DQ coherence associated with any N•−Y• interactions. The DQC of N•−N• pairs at 200 ns and 80 K is little affected as the T_2 of N• at this temperature is slightly faster than that at 25 K. The fraction of N•−N• pairs can be estimated to be <10% of N•−Y• pairs detected at 25 K.

To assign the measured distance of 48 ± 1 Å, we used the R1:R2 docking model[3] and considered possible distributions of N• and Y• within the complex (Figure 1b vs 1c). The simplest scenario based on our understanding of the mechanism of NDP reduction and N_3UDP inactivation of RNR is that the Y• in one R2 protomer gives rise to the N• on the symmetry-related R1 protomer, resulting in species b, Figure 1. If both protomers behaved in an identical fashion, then the N•−N• would be the major interacting species detected (Figure 1c). The inability to detect a modulation associated with this interaction (Figures 2 and 3) requires that the major species detected is b (Figure 1). The R1:R2 docking model and our knowledge of the structure of N• covalently bound to C225 on R1 allows an estimate of the distance between N• and Y• to be 47−50 Å. The measured distance is consistent with the docking model and rules out a large conformational change between R1 and R2 on active complex formation. The long distance supports the proposed radical migration through a pathway involving aromatic amino acids over a distance of 35 Å.[6] The data suggest a complex interplay of the R1, R2 subunits that permits ET from a single Y• in R2 to generate an N• in only one of the two active sites of R1. Further experiments with N_3UDP and with spin labels and spin traps on the ET pathway are in progress to understand this complex mechanism of radical initiation.

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