Supporting Information for

Equilibration of tyrosyl radicals (Y_{356}•, Y_{731}•, Y_{730}•) in the radical propagation pathway of the *E. coli* class Ia ribonucleotide reductase

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Supporting Methods:

Quantitation of the amount of [β-$^2H_2$]Y incorporated in α2 by LC-MS. Purified [β-$^2H_2$]Y-α2 (2 mg/ml) in 8 M urea (Sigma Aldrich), 50 mM ammonium bicarbonate (pH 7.8) was incubated with 10 mM DTT at 60 °C for 45 min. Cysteine residues of this protein were then alkylated by 22 mM iodoacetamide at room temperature (RT) in the dark for 1 h. Urea and excess reagent were removed by repeated concentration/dilution with 50 mM ammonium bicarbonate using a Microcon (Millipore) YM-10 centrifugal filtration device. Trypsin (Porcine pancreas, Sigma) was then added at an enzyme-to-substrate ratio (w/w) of 1:30, and incubated for 16 h at RT. The reaction was stopped by adjusting the pH to ~3 using 1% formic acid and the solution filtered through the Microcon YM-10 centrifugal filter to remove trypsin and any remaining undigested protein from the small peptides. The peptides were then analyzed by a nano flow high-performance liquid chromatography (HPLC) system (Eksigent Tempo) connected to an electrospray ionization mass spectometer, QSTAR Elite quadrupole time-of-flight mass spectrometer (AB Sciex). A reversed phase C_{18} capillary HPLC column (250 x 0.075 mm, Higgins Analytical) was used. The mass spectrometer was calibrated with the fragment ions of peptide Glu-fibrinopeptide B with a mass accuracy of < 25 ppm.

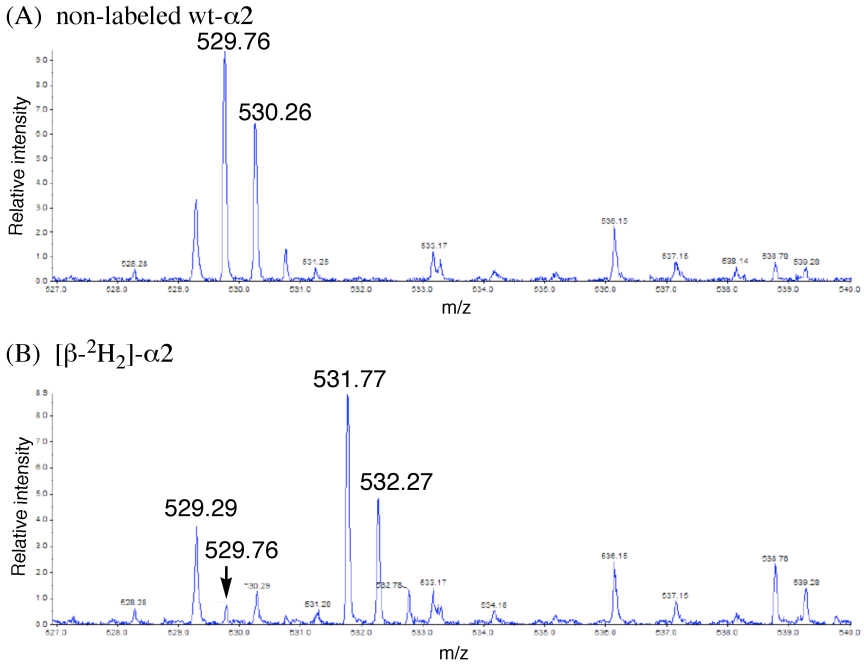

Figure S1. LC-MS analysis of tryptic peptides of non-labeled wt-α2 and [β-$^2H_2$]Y-α2. MS of peptide fragments containing Y_{730}- and Y_{731}-α2, TLYYQNTR, are shown for non-labeled wt-α2 (A) and [β-$^2H_2$]Y-α2 (B). The fragment was detected as the doubly charged ion with *m/z* of 529.76 ± 0.03 for non-labeled wt-α2 (A), which agrees with the calculated *m/z* of 529.77. In the [β-$^2H_2$]Y-α2 sample, a doubly charged ion at *m/z* 531.77 ± 0.05 was observed with the same retention time (B). This result is consistent with the calculated *m/z* of 531.77 for the peptide fragment with two [β-$^2H_2$]Ys. In B, a small signal with *m/z* 529.76 ± 0.03 that corresponds to the non-labeled peptide, and no signal that corresponds to the peptide with one [β-$^2H_2$]Y was observed. Based on the relative intensities of these peaks, the [β-$^2H_2$]Y incorporation was estimated to be > 92%. The sequence of peptides with *m/z* 529.76 observed in (A) and the peptide with *m/z* 531.77 in (B) were further analyzed by fragment ion MS to confirm that the observed signals are associated with the expected TLYQQNTR peptide.

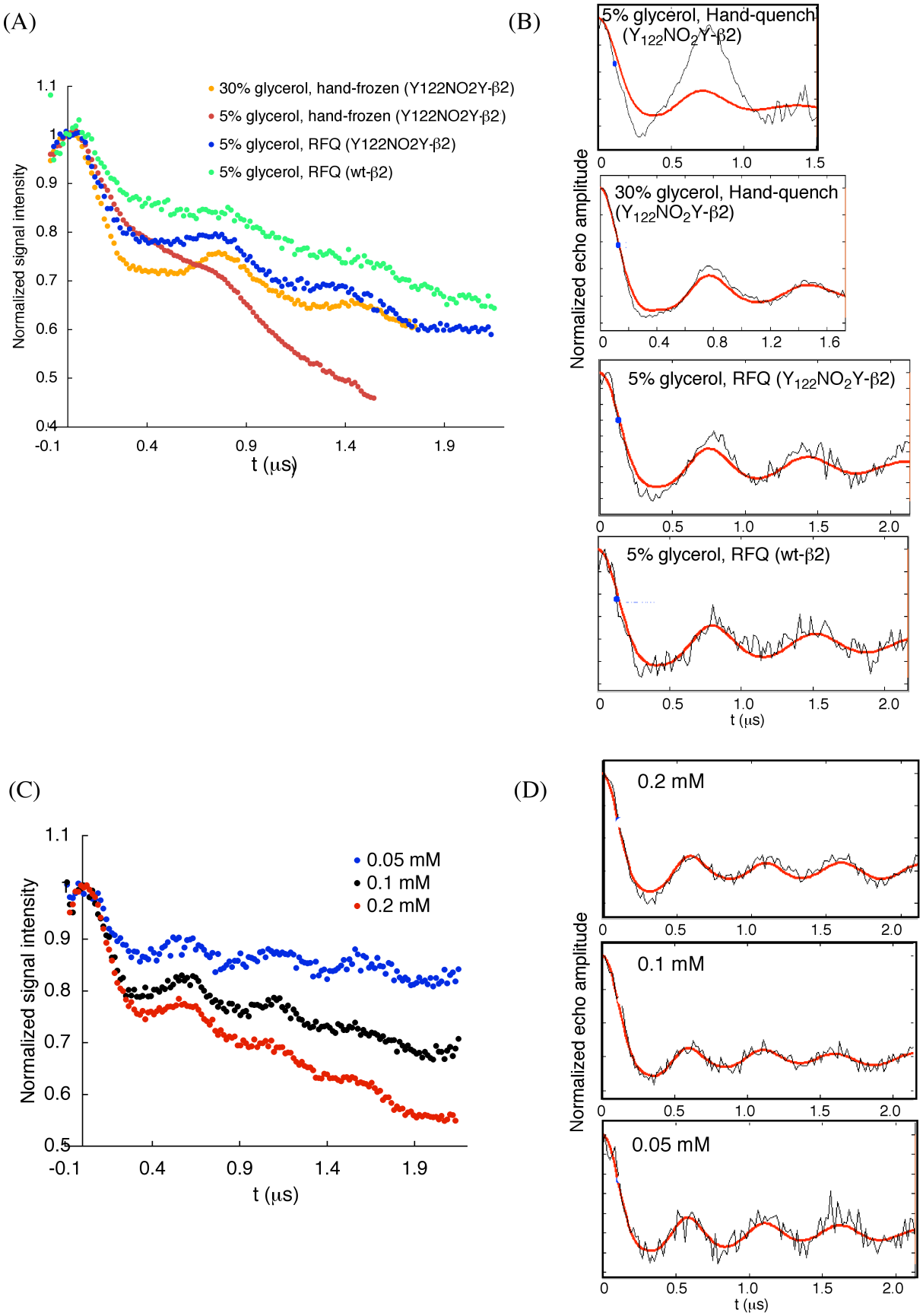
(A)
30% glycerol, hand-frozen (Y122NO2Y-β2)
5% glycerol, hand-frozen (Y122NO2Y-β2)
5% glycerol, RFQ (Y122NO2Y-β2)
5% glycerol, RFQ (wt-β2)
Normalized signal intensity
t (μs)
(B)
5% glycerol, Hand-quench (Y122NO2Y-β2)
30% glycerol, Hand-quench (Y122NO2Y-β2)
5% glycerol, RFQ (Y122NO2Y-β2)
5% glycerol, RFQ (wt-β2)
Normalized echo amplitude
t (μs)
(C)
0.05 mM
0.1 mM
0.2 mM
Normalized signal intensity
t (μs)
(D)
0.2 mM
0.1 mM
0.05 mM
Normalized echo amplitude
t (μs)

Figure S2. Effect of freezing method (A, B) and protein concentration (C, D) on the PELDOR spectra. (A) PELDOR spectra of 0.2 mM [NO_2Y_{122}•]-β2 prepared by hand-quenching with 5% glycerol (red) or 30% glycerol (yellow) and by rapid freeze quenching with 5% glycerol (blue), and with 0.2 mM wt-β2 prepared by rapid freeze quenching with 5% glycerol (green). All traces are normalized by the signal intensity at t = 0; (B) Spectra corrected by subtraction of monoexponential signal decay function and fit by Tikhonov regularization procedure[1]; (C) PELDOR spectra of rapid freeze quenched samples prepared with 0.2 (red), 0.1 (black) and 0.05 mM (blue) [NO_2Y_{122}•]-β2 and α2/CDP/ATP and quenched at 24 ms. All traces are normalized by the signal intensity at t = 0; (D) The analysis is as described in B.

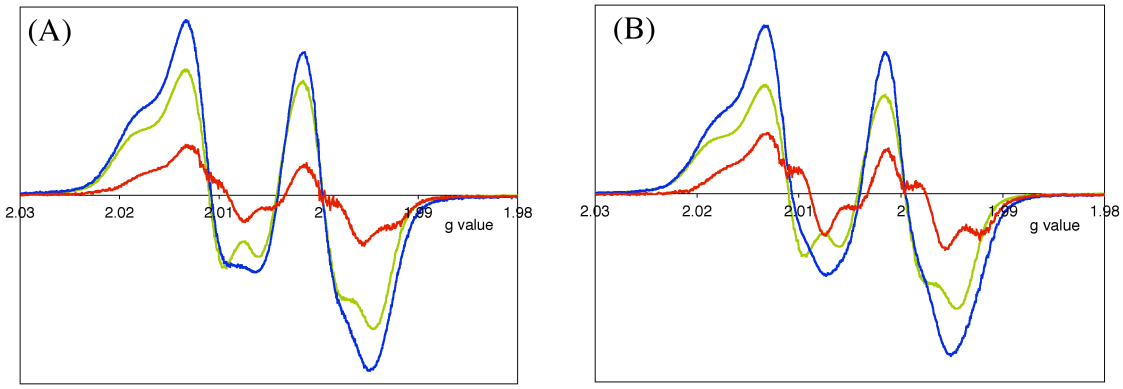

Figure S3. 9 GHz CW-EPR spectra of the reactions with [NO_2Y_{122}•]-β2 (0.1 mM), wt-α2 (0.1 mM), ATP (3 mM) and CDP (1 mM) at 25 °C and freeze quenched at (A) 8 or (B) 24 ms. The subtraction of NO_2Y• spectrum (green, 79 and 66% of total radical in (A) and (B), respectively) from the observed spectrum (blue) resulted in pathway Y• spectrum (red, 21 and 34 % in (A) and (B), respectively). Note that the NO_2Y• feature is broader than the Y• feature on the low field side facilitating subtractions.

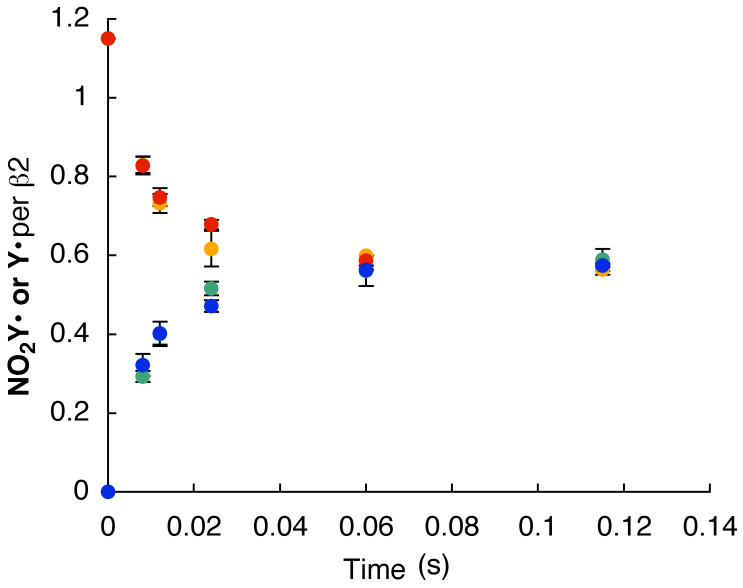

Figure S4. Time course of $NO_2Y\bullet$ reduction and Y• formation with [β-$^1H_2$]Y-α2 (blue, $NO_2Y\bullet$ and orange, Y•) and [β-$^2H_2$]Y-α2 (green, $NO_2Y\bullet$ and red, Y•) at 25 °C monitored by RFQ-EPR spectroscopy. Sequential mixing RFQ was carried out as described in Methods[2]. Each point represents an average of three replicates.

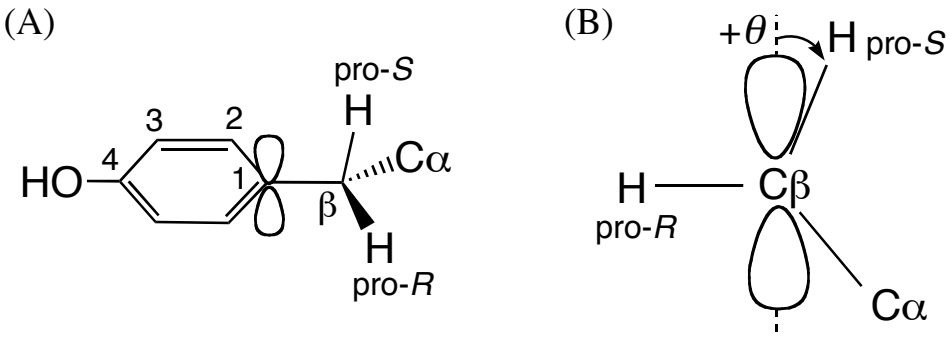

Figure S5. (A) Numbering for atoms of tyrosine. The p_z orbital on C1 is indicated. The amino and carboxy groups attached to Cα are omitted for clarity. (B) Definition of the ring rotation angle θ in tyrosine. θ (-90° ~ +90°) represents the dihedral angle between C_β-$H_{pro\text{-}S}$ bond and the p_z orbital on the C1 atom of the phenyl ring of Y.

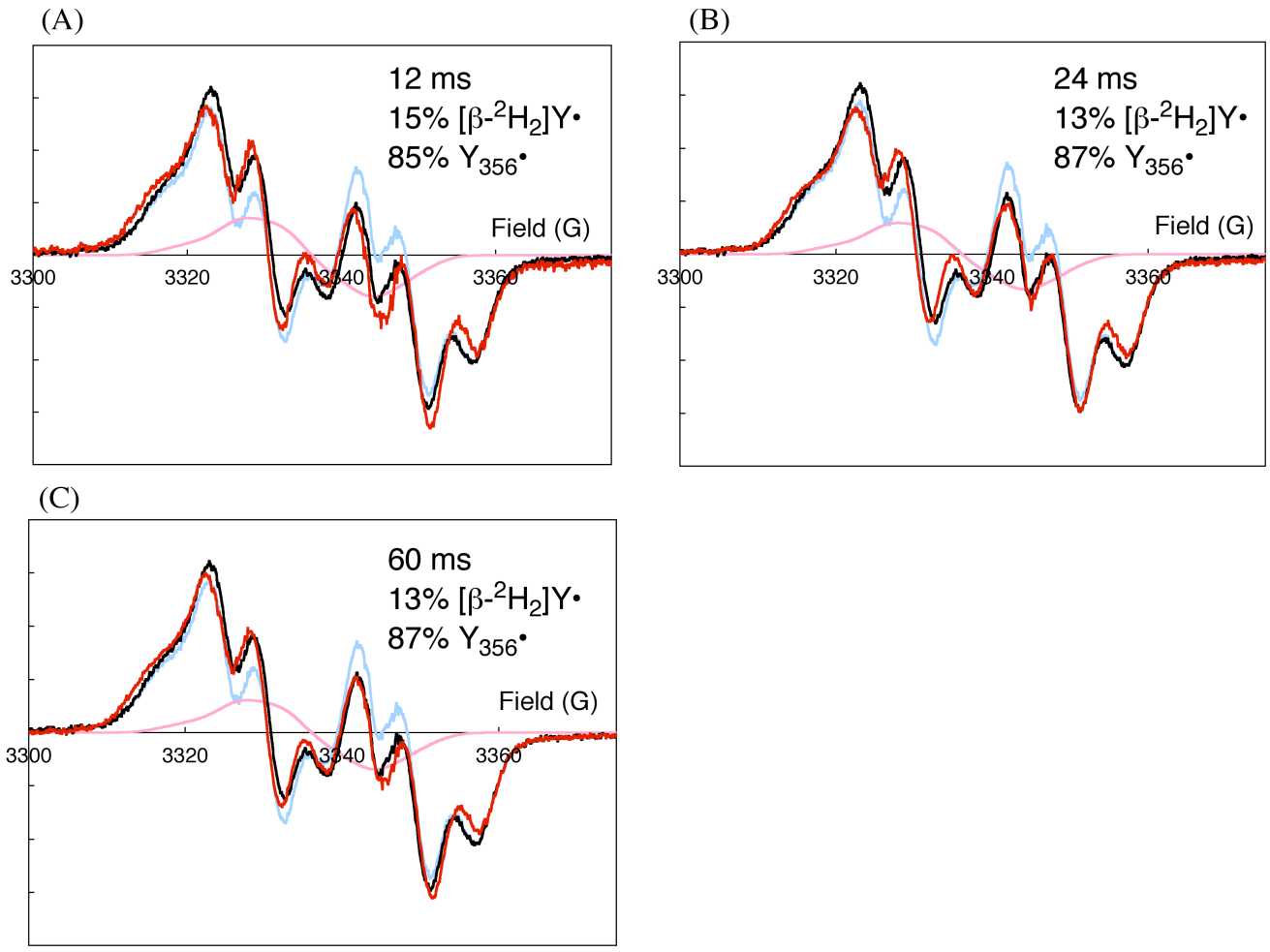

Figure S6. Quantitation of [β-$^2H_2$]Y• at 12, 24 and 60 ms at 25 °C. EPR spectra of the pathway Y• in the [NO_2Y_{122}•]-β2/[β-$^2H_2$]Y-α2/ATP/CDP reaction (red traces) were reconstructed using Y_{356}• observed in the [NO_2Y_{122}•]-β2/[Y_{731}F]-α2/ATP/CDP reaction (blue) and that of [β-$^2H_2$]Y• simulated using the parameters shown in Table 1 (pink). The sum of the Y_{356}• and [β-$^2H_2$]Y• spectra is shown in the black traces. To determine the amount of [β-$^2H_2$]Y•, the ratio of Y_{356}• and [β-$^2H_2$]Y• spectra was adjusted to minimize the difference between the red and the black traces. The ratios of Y_{356}• and [β-$^2H_2$]Y• determined by this analysis are indicated. Other details of the analyses are described in the main text.

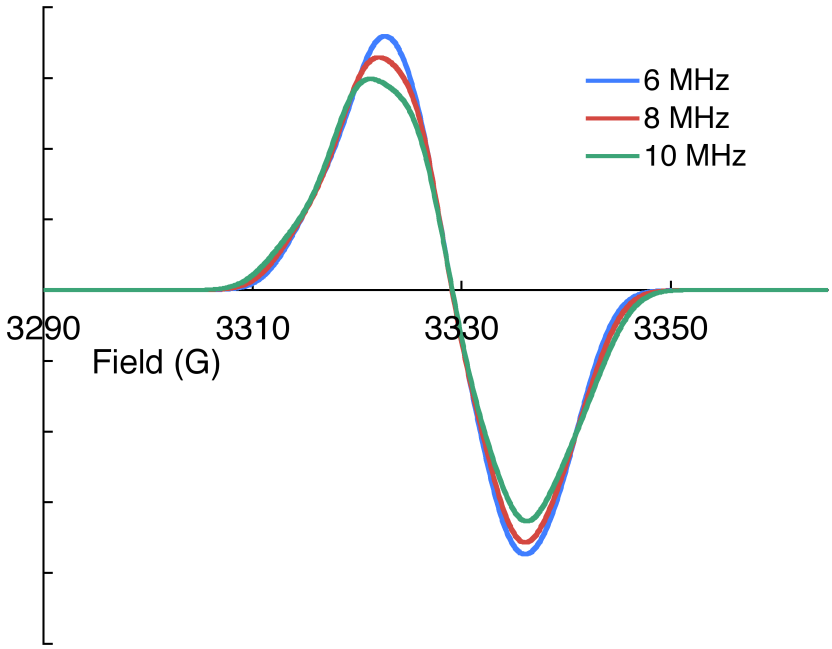

Figure S7. EPR spectral simulation for [β-$^{2}H_{2}$]Y• with hfcs for β-^{2}H of 6 (blue), 8 (red), and 10 MHz (green) with additional parameters summarized in Table 1 in the main text.

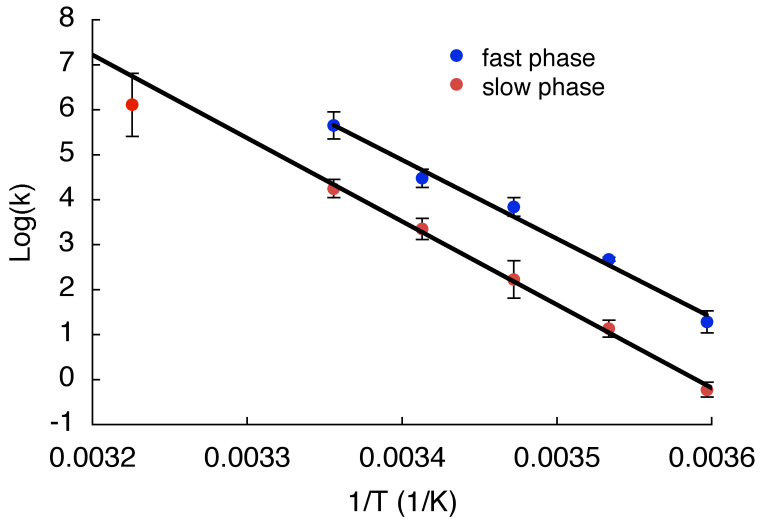

Figure S8. An Arrhenius plot of the NO_2Y• reduction in the reaction of [NO_2Y_{122}•]-β2/wt-α2/ATP/CDP monitored by SF-absorption spectroscopy at 460 nm as described previously[2]. Rate constants were determined from 4-7 traces. Briefly, $Fe(II)_2$-[NO_2Y_{122}]-β2 (40 μM, 5 Fe(II)/β2) in anaerobic 50 mM HEPES (pH 7.6) in syringe A was mixed with an equal volume of O_2-saturated 50 mM HEPES (pH 7.6) containing CDP (4 mM) in syringe B and aged for 0.5 - 2 s to maximize NO_2Y• formation in the incubation loop. The resulting solution was then mixed with equal volumes of wt-α2 (20 μM), ATP (6 mM), EDTA (2 mM) and $MgSO_4$ (30 mM) in 50 mM HEPES from syringe C. The final reaction mixture contained 10 μM [NO_2Y_{122}]-β2 and α2, 3 mM ATP, 1 mM CDP, 1 mM EDTA and 15 mM $MgSO_4$ in 50 mM HEPES. Experiments were carried out at 5, 10, 15, 20, 25 and 37 °C. In all cases, the kinetics were biphasic except for 37 °C, where the fast phase occurred within the mixing dead time. Lines are the linear least squares fit to log (k) = log (A) – E_a / (R x T), where k is a rate constant, E_a is the activation energy, R is the gas constant, and T is the temperature in K.

Table S1. Dihedral angles (θ) for Ys in α2.[a]

	Y_{730}[b]	Y_{731}[b]	PDB ID
E. coli wt	-67 °	+3 °	2X0X[3]
E. coli $Y_{730}NH_2Y$ mutant	-67 °	+82 °	2XO4[4]
Yeast	-58 °	+53 °	2WGH[5]
Human	-55 °	+61 °	2EUD[6]
Salmonella typhimurium	-68 °	+19 °	1PEQ[7]

[a] Dihedral angles (θ, see Figure S5 for definition) between the C_β-H bond and the p_z orbital on C1 of $Y_{730/731}$-α found in the crystal structure of class Ia α from *E. coli*, *S. cerevisiae* and human and class Ib α from *S. typhimurium*. [b] Numberings for *E. coli* α are used.

Reference

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