Supporting Information for

Direct Observation of a Transient Tyrosine Radical Competent for Initiating Turnover in a Photochemical Ribonucleotide Reductase

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List of Abbreviations: α2, large subunit of RNR containing substrate and effector binding sites; ATP, adenosine-5'-triphosphate; β2, small subunit of RNR containing the diiron-tyrosyl radical cofactor; CDP, cytidine-5'-diphosphate; DMF, dimethylformamide; DNA, deoxyribonucleic acid; HPLC, high-performance liquid chromatography; PCET, proton-coupled electron transfer; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; NADPH, nicotinamide adenine dinucleotide; PELDOR, pulsed electron-electron double resonance; R2C19, 19-mer, C-terminal peptide tail of β2; [Re], $Re(bpy)(CO)_3CN$; RNR, class I *E. coli* ribonucleotide reductase; TA, transient absorption; TEAB, triethylammonium bicarbonate; TR, *E. coli* thioredoxin; TRR, *E. coli* thioredoxin reductase; Tris, *tris*(hydroxymethyl)aminomethane

Materials. Trifluoroacetic acid (TFA), adenosine 5′-triphosphate (disodium salt from bacteria, ATP), cytidine 5′-diphosphate (sodium salt from yeast, CDP), piperidine, and diisopropylethylamine (DIPEA), were purchased from Sigma-Aldrich. O-(7-Azobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), N-[(9H-fluoren-9-ylmethoxy)-carbonyl]-L-phenylalanine (Fmoc-F-OH) and O-(1,1-dimethylethyl)-N-[(9H-fluoren-9-ylmethoxy)carbonyl]-L-tyrosine (Fmoc-Y(*t*Bu)-OH) were purchased from Applied Biosystems. 1-Hydroxybenzotriazole (HOBt) was obtained from NovaBiochem. [5-^{3}H]-Cytidine diphosphate was purchased from Amersham and purified as described below. Calf-intestine alkaline phosphatase (10 U/μL) was purchased from New England Biolabs. Piperidine was freshly distilled from KOH under N_2 prior to use. Fmoc-3,5-F_2Y was made in two steps from 2,6-difluorophenol, pyruvate, and ammonia using pyruvate formate lyase as previously described.[1,2] $Re(bpy\text{-}COOH)(CO)_3CN$ was prepared as previously described.[3]

Purification of [5-^{3}H]-CDP. 250 μCi of [5-^{3}H]-CDP (Amersham, 24 μCi/nmol, 97.9% radiochemical purity) was diluted to a specific activity of ~0.018 μCi/nmol with cold cytidine diphosphate (Sigma). The diluted stock solution was then purified over a diethylaminoethyl (DEAE)-Sephadex A25 column. The resin was purchased in the chloride form from Sigma-Aldrich, swelled in 10 mM triethylammonium bicarbonate (TEAB) solution, and poured into a 60 mL column. The resin was washed with 5 column volumes of 1M TEAB solution, followed by five column volumes of deionized water. The stock [5-

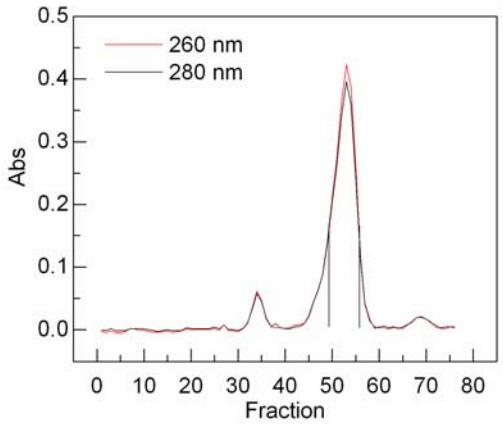

Figure S1. *DEAE ion exchange purification of [5-^{3}H]-CDP. Fractions between the vertical lines were collected and pooled.*

1. Seyedsayamdost, M. R.; Reece, S. Y.; Nocera, D. G.; Stubbe, J. *J. Am. Chem. Soc.* **2006**, *128*, 1562.
2. Seyedsayamdost, M. R.; Reece, S. Y.; Nocera, D. G.; Stubbe, J. *J. Am. Chem. Soc.***2006**, *128*, 1569.
3. Reece, S. Y.; Nocera, D. G. *J. Am. Chem. Soc.* **2005**, *127*, 9448.

^{3}H]-CDP solution was then slowly loaded onto the column and eluted with a 0 → 600 mM TEAB gradient (450 mL × 450 mL). Fractions (400 drops) were collected automatically by a fraction collector and every other fraction analyzed by a 96 well plate UV-vis absorption spectrometer at 260 and 280 nm. Figure S2 plots the absorption trace for this column separation; fractions were pooled between the two horizontal lines (49-56). The solvent and TEAB were removed *in vacuo*, and the resulting product dissolved in deionized water. The entire column separation procedure was repeated, yielding a 20 mM stock solution with 11,100 Cpm/nmol (99.95% radiochemical purity as assayed by the C/dC ion exchange separation procedure[4,5]).

Synthesis of [Re]-(F/Y/3,5-F_2Y)LVGQIDSEVDTDDLSNFQL [[Re]-(F/Y/3,5-F_2Y)-R2C19]. Solid phase peptide synthesis (SPPS) using Fmoc-protected amino acids was employed to extend the Fmoc-R2C19-PEG-PS resin bound peptide, which was available from a previous study.[6] For the syntheses of the [Re]-(F/Y/3,5-F_2Y)-R2C19 peptides, typically 250 mg of the Fmoc-R2C19-PEG-PS resin bound peptide (0.2 mmol/g) was loaded into a 10 mL Bio-Rad Poly-Prep column containing a porous 30 μm polyethylene bed in the bottom to hold the resin. The N-terminal Fmoc protecting group was cleaved by shaking the resin in a solution of 3.6 mL 0.1 M HOBt in 20% piperidine/DMF for 3 × 8 min using a Fisher Scientific Vortex Genie 2 (VWR). The resin was then washed with 3 × 4 mL of DMF and CH_2Cl_2. Fmoc-F-OH/ Fmoc-Y(*t*Bu)-OH/ Fmoc-3,5-F_2Y was then coupled to the free N-terminus by shaking the resin for 2 × 70 - 80 min in a solution of 0.5 M amino acid, 0.45 M HATU and 1 M DIPEA; the volume was adjusted accordingly such that the amino acid was in 6 - 8 fold excess. The Re(bpy-COOH)$(CO)_3$CN chromophore was then coupled in a similar manner with some modifications: the coupling solution consisted of 27 mg of Re(bpy-COOH)$(CO)_3$CN in 1.5 mL DMF with 77 mg of HATU and 100 μL of DIPEA due to limited solubility of the chromophore. The coupling reaction was repeated to ensure high yields of the desired product. Cleavage of the peptide from the resin was carried out by shaking in 5 mL of 95% TFA, 2.5% triisopropylsilane, 2.5% water for 4 h. The resin was then washed for 2 × 1 min with 2 mL of TFA. The cleavage cocktail and washings were combined, evaporated under a stream of N_2, and taken up in 15 mL of ether to precipitate the crude peptide, which was pelleted in a centrifuge and the ether decanted. The precipitate was dissolved in 0.1 M ammonium bicarbonate HPLC buffer via sonication over several minutes.

Peptide Purification and Characterization. Peptides were purified by reversed phase HPLC using the system previously described.[7] Samples were manually injected onto a preparative Waters XTerra MS C-8 column (30 × 100 mm), which had been previously equilibrated with 10% acetonitrile/10 mM potassium phosphate buffer (pH 6). A linear gradient of 10 → 50 % acetonitrile vs. phosphate buffer over 50 min at a flow rate of 10 mL/min was

4. Steeper, J. R.; Steuart, C. D. *Anal. Biochem.* **1970**, *34*, 123.
5. Booker, S.; Licht, S.; Broderick, J.; Stubbe, J. *Biochemistry* **1994**, *33*, 12676.
6. Yee, C.S.; Seyedsayamdost, M. R.; Chang, M. C. Y.; Nocera, D. G.; Stubbe, J. *Biochemistry* **2003**, *42*, 14541.
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Table S1. Characterization of Peptides

Peptide	t_R (min)[a]	MW Calcd (m/z)	MW Found (m/z)	IC_{50}[d] (μM)
[Re]-F-R2C19	25.5	2748	2721[b]	-
[Re]-Y-R2C19	23.8	2764	2737[b]	8
[Re]-3,5-F_2Y-R2C19	19.5	2800	1399[c], 2772[b]	-

[a] Retention time (t_R) from analytical HPLC trace. [b] $[M - CN]^+$ ion from MALDI-TOF MS. [c] $[M - 2H]^{2-}$ ion from ESI-FT MS. [d] Concentration of peptide at 50% RNR inhibition.

used to elute the peptides. The eluant absorbance was monitored at 355 and 210 nm, where the [Re] chromophore and peptide amide bond absorb, respectively. Fractions containing the desired product were collected by hand, pooled, and reinjected onto the C-8 prep column which had been equilibrated with 0.1 M ammonium bicarbonate. The loaded column was rinsed with 5 column volumes of ammonium bicarbonate buffer, and the peptide eluted with 1:1 acetonitrile / 0.1 M ammonium bicarbonate. The eluant was collected by hand, lyophilized, taken up in 50 mM Tris buffer at pH 7.5, combined, and stored at –80 °C. Analytical HPLC was employed to confirm the purity of the peptides. The samples were manually injected onto an analytical Waters XTerra MS C-8 (4.6 × 100 mm) column and eluted with a 10 → 65% acetonitrile vs. 10 mM phosphate buffer (pH 6) over 45 minutes at a flow rate of 1 mL/min. The molecular weight (MW) of the peptide was characterized by MALDI-TOF mass spectrometry as described below. The HPLC retention times (t_R) and MALDI-TOF m/z ratios for each peptide are listed in Table S1. Analytical HPLC traces for each peptide were recorded at 210 and 355 nm to confirm purity, and

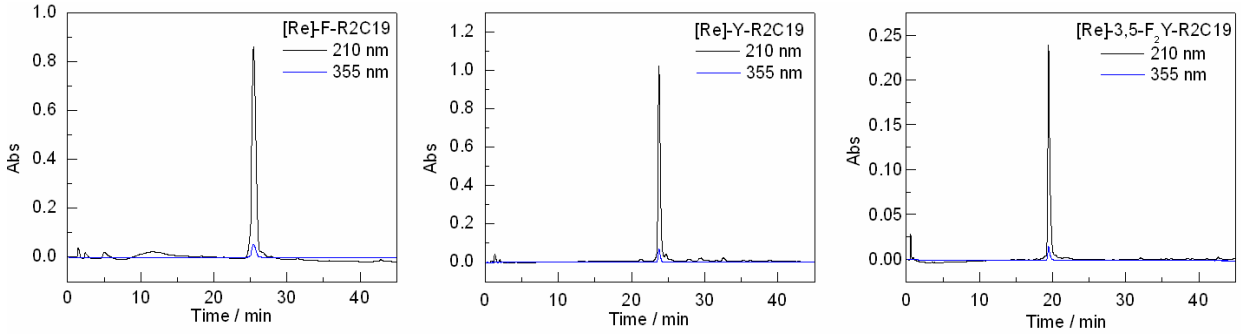

Figure S2. Analytical HPLC trace for [Re]-F-R2C19 (left), [Re]-Y-R2C19 (middle), and [Re]-3,5-F_2Y-R2C19 (right) obtained by monitoring absorbance at 210 (—) and 355 nm (—) using 10→65% MeCN/ 10 mM KP_i (pH 6) linear gradient over 45 min (1 mL min^{-1} flow rate).

are shown in Figure S3. The concentration of each peptide stock solution was estimated by UV-vis absorbance using the known $\varepsilon_{355\ nm}$ = 5300 M^{-1} cm^{-1} for the [Re]-3,5-F_2Y-OMe dipeptide.

Isolation, Purification and Pre-reduction of α2. *E. coli* α2,[8] and Y731F-α2[9] were isolated by standard procedures. To remove contaminating β2, these proteins were further purified using a POROS HQ/20 anion exchange column (Applied Biosystems) on a BIOCAD Sprint Perfusion Chromatography System (Applied Biosystems). The column was loaded with α2 (10 - 15 mg) and washed with Tris buffer (50 mM, pH 7.6) for 5 min. α2 was eluted with a linear gradient of 0 - 700 mM NaCl over 30 min at a flow rate of 4 mL/min. Fractions were collected by hand and concentrated on a YM-30 membrane (Millipore). This procedure reduced background turnover of CDP 7 - 8 fold. To pre-reduce α2, ~30 mg were incubated with 30 mM DTT for 30 min at room temperature. Hydroxyurea, ATP and CDP were added to final concentrations of 30 mM, 3 mM and 1 mM, respectively, and the incubation continued for an additional 20 min. Another 10 mM DTT was added, and the mixture incubated for 10 min and desalted on a G-25 Sephadex column (~35 mL, 1.5 × 23 cm) pre-equilibrated in 50 mM Tris, 15 mM $MgSO_4$, pH 7.6.

α2 Activity Assay and Competitive Inhibition Assay for Binding of Peptide to α2. α2 (0.1 μM, specific activity = 1900 nmol min^{-1} mg^{-1}), β2 (0.2 μM, specific activity = 6800 nmol min^{-1} mg^{-1}), TR (30 μM), TRR (0.5 μM), NADPH (0.2 mM), CDP (1 mM), and ATP (1.6 mM) were combined in 50 mM HEPES, 15 mM $MgSO_4$, 1 mM EDTA buffer at pH 7.6. Enzyme activity was measured by the consumption of NADPH, which was monitored by the decrease in absorbance at 340 nm. This was then repeated with peptide concentrations ranging from 1 - 150 μM. These data are plotted in Figure 2 (main text).

Single Turnover Assays for Photoinitiated Nucleotide Reduction in Peptide-α2 Complexes. The assays were performed by a method modified from that previously described.[7] For the data in Figure 2, [5-^{3}H]-CDP (0.75 mM, 11,100 Cpm/nmol) substrate was used. FPLC purified, pre-reduced α2 (20 μM, specific activity = 1800 nmol min^{-1} mg^{-1}), peptide (200 μM), ATP (3.0 mM), and labeled-CDP in 50 mM Tris, 15 mM $MgSO_4$ buffer (pH 7.5 unless otherwise indicated) were added to a 1 cm quartz micro-cuvette with total solution volume of 200 μL. The data in Figure 2 were also collected in the presence of 20% glycerol, which served to increase turnover yield and reproducibility of the data. The reaction was initiated by the addition of CDP, quickly mixed, spun down in a minicentrifuge (< 10 s, 2000 g) to consolidate the liquid, and pipetted into the cuvette. Samples were irradiated at room temperature with the focused light from a 1000 W Xe arc lamp equipped with a 348 nm long pass filter. The cuvette was contained in a pyrex beaker filled with water at 25 °C to regulate the temperature during photolysis. Fractions (60 μL) were removed at 2, 5 and 10 min intervals and immediately quenched by heating in a boiling water bath for 2 min. The precipitated protein was then spun down for 10 min in a minicentrifuge at 20,000 *g*. The supernatant (50 of the 60 μL) was transferred to a new Eppendorf tube, diluted with 14 units of alkaline phosphatase and 120 nmol of carrier dC to a

8. Salowe, S.; Bollinger, J. M., Jr.; Ator, M.; Stubbe, J.; McCraken, J.; Peisach, J.; Samano, M. C.; Robins, M. J. *Biochemistry* **1993**, *32*, 12749.
9. Salowe, S.; Stubbe, J. *J. Bacteriol.* **1986**, *165*, 363.

final volume of 170 μL, and incubated at 37 °C for 2 h in a sealed Eppendorf vial. dC was then separated from C and quantitated as previously described.[4,10]

The data shown in Figure 2 are corrected by subtracting for background counts due to radiochemical impurities, which were quantified with dark control experiments and found to be less than 20% of the maximum counts reported for the photolyzed samples. Each time point is an average of two separate experiments. The data are reported as percent of the maximum turnover equivalents per α (1.0 eq dCDP), which was quantified by incubation of α2 with β2 as previously described.[7] This experiment was also performed at pH 8.2, yielding a maximum of 0.85 eq dCDP per α.

Physical Measurements. ESI-FT mass spectrometry was performed with a Brüker Daltonics APEXII instrument housed in the DCIF. MALDI-TOF mass spectrometry was performed with a Brüker Omniflex instrument in the DCIF using α-cyano-4-hydroxycinnamic acid as the matrix. The instrument was calibrated in positive ion mode with a quadratic polynomial using a mixture of angiotensin II (1046.5423), P14R synthetic peptide (1533.8582), and ACTH fragment 18-39 (2465.1989) (Sigma). All peptides synthesized were analyzed in *positive* ion mode as the $[M\text{-}CN]^+$ ion for MALDI-TOF or in negative ion mode as the $[M - 2H]^{2-}$ for ESI-FT MS.

Nanosecond transient absorption (TA) measurements were made with the third harmonic (355 nm) of an Infinity Nd:YAG laser (Coherent) running at 10 Hz. Thus system was extensively modified from that previously described.[11] Probe light was provided by a pulsed 75 W Xe-arc lamp (Photon Technologies Incorporated). The lamp was pulsed with 5 A current (1 ms pulse width) and triggered externally at 10 Hz. The signal light passed through a Triax 320 spectrometer, where it was dispersed by a 300 × 500 blazed grating and collected with either an intensified gated CCD camera (ICCD, CCD 30-11, Andor Technology, 1024 × 256 pixels, 26 μm^2) for TA spectra or a photomultiplier tube (PMT) for TA kinetics at a single wavelength. PMT outputs were collected and averaged with a 1 GHz oscilloscope (LeCroy 9384CM). A TTL pulse synchronized with the Q-switch of the Infinity laser was delayed 99 ms before triggering the pulser for the probe light. Electronic delays were created with SRS DG535 delay generators (Stanford Research Systems). These delay boxes, in combination with electronic shutters (Uniblitz), were used to create the pulse sequence illustrated in Figure S5.

10. Booker, S.; Licht, S.; Broderick, J.; Stubbe, J. *Biochemistry* **1994**, *33*, 12676.
11. Loh, Z.-H.; Miller, S. E.; Chang, C. J.; Carpenter, S. D.; Nocera, D. G. *J. Phys. Chem. A* **2002**, *106*, 11700.

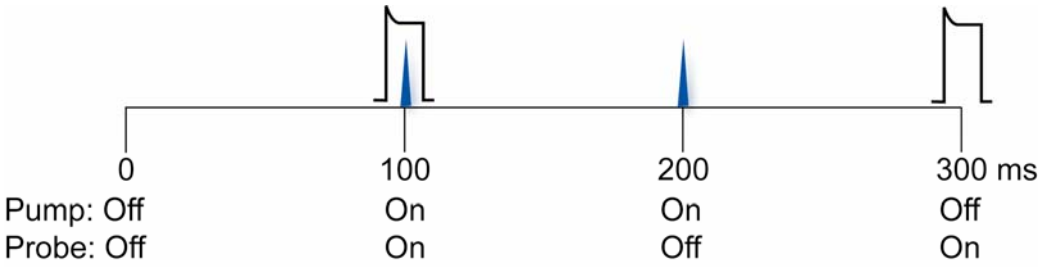

Figure S3. Timing of the nanosecond TA instrument running at 10 Hz.

To produce a TA spectrum, the series of four spectra were collected: I_B (pump off/probe off), I (pump on/probe on), I_F (pump on/probe off), and I_0 (pump off/probe on). Transient spectra were corrected for fluorescence and background light using these spectra and the calculation: $\Delta OD = \log[(I_0 - I_B)/(I - I_F)]$. For experiments involving α2, the spectra reported are an average of 125 of the four-spectra sequences. Sample sizes were typically 200 μL in a 2 × 10 mm cuvette containing a Teflon-coated mini-stirbar. Both the white light and pump beams were focused and overlapped to pass through the 2mm-wide window of the cuvette, providing a total pathlength of 1 cm. To provide optimal beam overlap, the pump beam was reflected off a small mirror in front of the collimating lense for the probe beam after the sample, as illustrated in Figure S6.

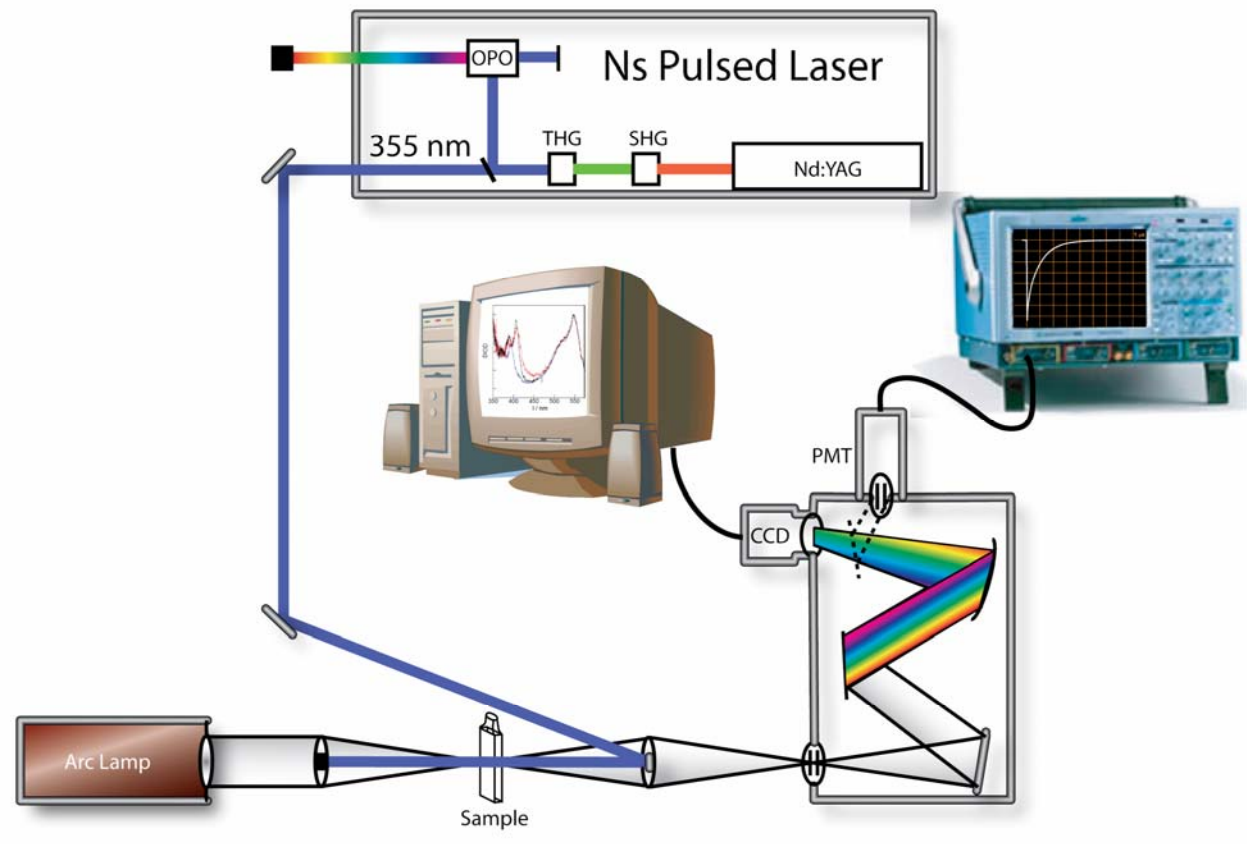

Figure S4. Diagram of the nanosecond TA instrument running at 10 Hz.

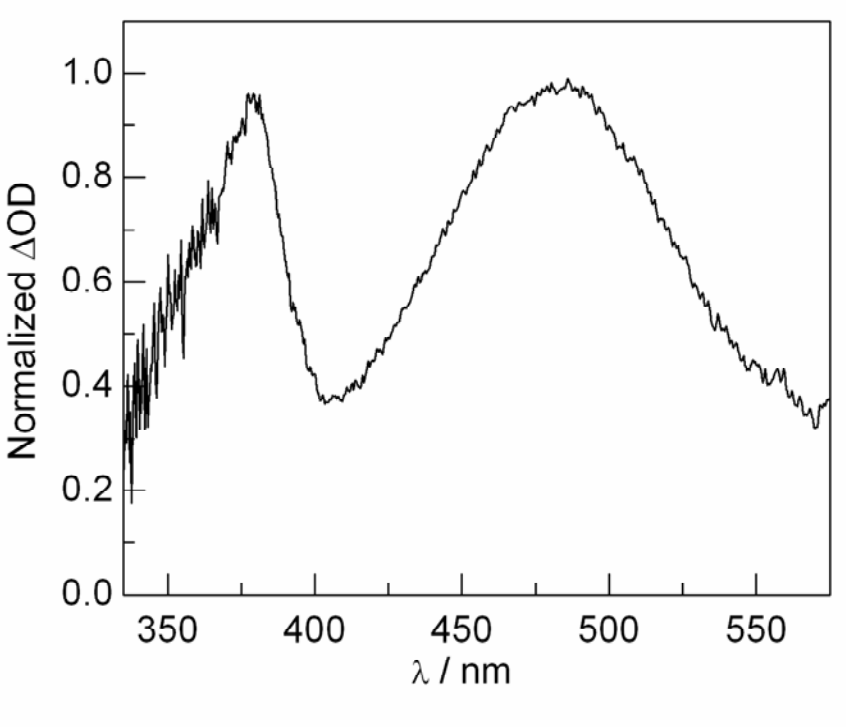

Figure S5. Theoretical calculated TA spectrum consisting of 87 % ^{3}[Re]* excited state and 13 % [Re]^{0}-3,5-F_2Y• charge separated state.