Supporting Information

Deciphering Radical Transport in the Catalytic Subunit of Class I Ribonucleotide Reductase

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Source of Materials and Reagents

Acros: 2,3,5-trifluorophenol; Strem: rheniumpentacarbonyl chloride [$Re(CO)_5Cl$] and Hexaaminеruthenium(III) Chloride [$Ru(NH_3)_6Cl_3$]; NovaBioChem: Fmoc-succinimide and 2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU); Sigma: Lactate dehydrogenase (LDH) and adenosine-5′-triphosphate (ATP); Aldrich: sodium borate ($Na_2B_4O_7$), DL-dithiothreitol (DTT), β-mercaptoethanol (β-ME), ampicillin sodium salt (Amp), pyridoxal-5′-phosphate (PLP), phenylmethylsulfonyl fluoride (PMSF), streptomycin sulfate (strep), kanamycin sulfate (Km), ethyl acetate (EtOAc), dichloromethane (DCM), diethyl ether (Et_2O), trifluoroacetic acid (TFA), *N,N*-dimethylformamide (DMF), ethanol (EtOH), acetonitrile (MeCN), diisopropylethylamine (DIPEA), triethylamine (Et_3N), triisopropyl-silane (TIPS). Invitrogen: SOC Media; Mallinckrodt: ethylenediaminetetraacetic acid (EDTA), potassium phosphate (KPi); EMD: magnesium sulfate ($MgSO_4$), sodium chloride (NaCl), tris(hydroxymethyl)aminomethane (Tris), ammonium sulfate [$(NH_4)_2SO_4$], VWR (BDH): glycerol; BD Biosciences: Luria-Bertani media.

Sequence of Mutagenesis Primers

pET-*nrdA*(Y731F)
Forward: 5′-G GTC AAA ACA CTG TAT TTT CAG AAC ACC CG-3′
Reverse: 5′-CG GGT GTT CTG AAA ATA CAG TGT TTT GAC C-3′

pET-*nrdA*(Y730F)
Forward: 5′-C GGG GTC AAA ACG CTG TTT TAT CAG AAC ACC CGT G-3′
Reverse: 5′-C ACG GGT GTT CTG ATA AAA CAG CGT TTT GAC CCC G-3′

pET-nrdA(C439S)
Forward: 5′-G CGT CAG TCT AAC CTG TCC CTG GAG ATA GCC C-3′
Reverse: 5′-G GGC TAT CTC CAG GGA CAG GTT AGA CTG ACG C-3′

Tools, Instrumentation and Methods

Measurement of small volumes. All small volume (< 1 mL) measurements were made using VWR EHP micropipettors that had been calibrated within one year (Pipette Calibration Services, Newton Highlands, MA). The appropriate pipette was used for each volume range, as follows: 1–10 μL (P10), 10–20 μL (P20), 20–100 μL (P100), 100–200 μL (P200), 200–1000 μL (P1000). For protein samples with a volume between 200 and 400 μL, a P200 was used twice to ensure greater accuracy, with a fresh tip for each draw, as described in the manuscript.

Protein chromatography. All protein chromatography was performed under gravity flow at an ambient temperature of 4 °C, with flow rates listed for each column prepared. Fractions were collected with a GE Healthcare Frac-920 collector as described for each column prepared.

HPLC. High performance liquid chromatography was performed on an Agilent 1200 series instrument fitted with an inline Diode Array UV Absorption Detector and an inline Fluorescence detector. Analytical chromatography was accomplished on a Phenomenex Kinetix XB-C18 4.6 × 100 mm reversed-phase column fitted with a guard cartridge. Separation gradients are listed with results. Semi-preparative scale purifications were accomplished on a Waters 19 × 150 mm C18 reversed-phase column fitted with a guard cartridge. For these runs, the instrument was set to collect fractions for designated time periods at 0.5 min intervals.

NMR. ^{1}H and ^{13}C spectra were measured with a Bruker AVQ-400 (400 MHz) spectrometer. Chemical shifts are reported as δ in units of parts per million (ppm) relative to standards (as listed). Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), m (multiplet), br (broadened), or app (apparent). Coupling constants are reported as a J value in Hertz (Hz). The number of protons (n) for a given resonance is indicated nH, and is based on spectral integration values.

HR MALDI-TOF MS. Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry was performed on a Bruker OmniFlex system in positive reflector detection mode. α-cyanohydroxycinnamic acid was used as a matrix. Solutions of matrix were prepared day-of as a saturated solution in 1:1 MeCN:H_2O + 0.5% v/v TFA. The matrix and sample solutions were mixed at a ratio of 9:1 matrix:sample, by volume, and spotted on a stainless steel target and allowed to dry before analysis. Spectra were collected as an average of 500 shots. Peaks were calibrated externally each day using the proteomass peptide MALDI-MS calibration kit (Sigma) by following the manufacturer's instructions. For each spectrum, four peaks were used to generate the reference file.

SDS-PAGE. For protein analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a Mini-Protean apparatus from Bio-Rad, following the general protocol of Laemmli.[1] Gels were pre-cast 7.5% TGX (Bio-Rad). All protein electrophoresis samples were heated for 2 min at 200 °C in the presence of DTT to

[1] Laemmli, U. K. *Nature* **1970**, *227*, 680–685.

ensure reduction of any disulfide bonds. Commercially available markers (Dual Color Protein Plus, Bio-Rad) were applied to at least one lane of each gel for assignment of apparent molecular masses. Gels were stained with Coomassie Brilliant Blue R-250 before imaging. Gel images were collected with a Bio-Rad Gel Doc 2000, saved using Quantity One version 4.4.0 software, and exported without modification as 16-bit tif images.

Fluorescence spectroscopy. Steady state emission spectra were recorded on an automated Photon Technology International (PTI) QM 4 fluorimeter equipped with a 150-W Xe arc lamp and a Hamamatsu R928 photomultiplier tube. Samples (typically 75–100 µL) were collected with 1 s integration time and 1 nm step size.

UV–vis spectroscopy. UV–vis spectra were recorded on a Varian Cary 5000 UV/VIS/NIR spectrometer. Samples (typically 75–100 µL) were measured as a baseline-subtracted spectrum with 0.1 s integration and 1 nm observation slit width at each data point.

Radioactivity scintillation counting. Measurement of [^{3}H] decay counts were collected on a Beckman LS 6500 Scintillation counter. Each counted solution was a mixture of 9 mL of scintillation fluid and 1 mL of sample solution, which was vigorously vortexed before counting for 10 min per sample.

Time-resolved spectroscopic laser system. Transient measurements were recorded on a home-built system whose timing schematic is shown in Figure S8. Pump light was provided by the third harmonic (355 nm) of a Quanta-Ray Nd:YAG laser (Spectra-Physics) running at 10 Hz with a power output set to 3 mJ/pulse using a combination of the instrument's power knob and a 0.2 OD filter. Laser power was measured using a Coherent J-25MB-HE meter coupled to a Coherent Labmax Top. Probe white light was the output of a 75 W Xe-arc lamp (A1010B arc lamp, LPS-220B power supply, Photon Technologies Incorporated). A TTL pulse synchronized with the Q-switch of Infinity laser was used to generate the 0 time point of the system. All timed delays were created with SRS DG535 delay generators (Stanford Research Systems). Uniblitz electronic shutters model VS14S2Z0R3 (Vincent Associates, Rochester, NY) were used to create the pulse sequence illustrated in Figure S1. Both the white light and pump beams were focused and overlapped at a 15° angle to pass through the cylindrical bore of a flow cuvette (585.3/Q/10/Z15/AR, Starna Inc., Atascadero, CA), providing a total pathlength of 1 cm. The sample was flowed from a 1.5 mL eppendorf tube, through the flow cell, then the pump (Cole-Parmer L/S 7518-00), the inline filter (see below) and back to the eppendorf tube, through a combination of pump tubing (Masterflex L/S 13) and the included fittings for the flow cell. The resulting signal light was passed into a Triax 320 spectrometer with a slit width of 0.4 mm (correlating to 5 nm).

Transient absorption (TA) spectroscopy. Signal light entering the spectrometer was first dispersed by a 300 × 250 blazed grating. The dispersed light was recorded on an intensified gated CCD camera (ICCD, CCD 30-11, Andor Technology, 1024 × 256 pixels, 26 μm^2), whose delay and exposure were timed from the output of the SRS DG535 delay generator. A series of four spectra were taken using the on/off state of the pump and probe shutters: I_F (pump on/probe off), I (pump on/probe on), I_B (pump off/probe off), and I_0 (pump off/probe on). Spectra were corrected for fluorescence and background light using these data by the equation:

$$\Delta OD = -log\left(\frac{I-I_F}{I_0-I_B}\right) \quad \text{(S1)}$$

Transient absorption kinetics. In this case, signal light entering the spectrometer was first dispersed by a 300 × 500 blazed grating, and selected wavelengths were passed through an output with a slit width of 0.4 mm (5 nm). The resulting transient pulses were recorded with a photomultiplier tube (Hammamatsu 928A) poised at 1.00 kV. PMT outputs were collected and averaged with a 1 GHz oscilloscope (LeCroy 9384CM), whose trace was captured through a GPIB interface on a PC. The oscilloscope was triggered by the TTL input of a photodiode that was aligned to respond to the backscattered light from the flow cuvette. Unlike the TA spectra, both the pump and probe light shutters were open for the duration of the measurement. To perform a specific number of scans, we first opened the probe shutter and the spectrometer slits to pass light. The software was then set to record a desired interval, the oscilloscope memory was cleared, and finally the pump shutter was opened, which triggered the photodiode to begin recording. After accumulating the desired number of scans, the software triggered a data save event.

Time resolved emission kinetics. Time resolved emission data were generated with the same system as the transient absorption kinetics above, but without the white light from the arc lamp.

Commercial and open-source software. Graphs were generated from Cartesian data pairs in Origin (v. 8.0; OriginLab Corp, Northhampton, MA, www.originlab.com). 16-bit .tif gel images were first processed with Adobe Photoshop (v. CS5, Adobe Systems Inc., San Jose, CA, www.adobe.com) by setting the image levels automatically, then cropping the pixels and saving. ImageJ (version 1.43u, NIH, http://rsb.info.nih.gov/ij/) was then used to subtract the background using a 50-pixel rolling-ball, and protein purity was calculated from integration of the area under vertical slices of the optical density plots of the gel bands. Mass spectra were transformed from the collected raw data with the Bruker

CompassXport tool (v. 3.0; Bruker Daltonics, Inc., Billerica, MA, www.bdal.com) by mmass software[2] (v. 3.9, www.mmass.org), which was further used for mass calibration and export. NMR analysis was performed with SpinWorks (v. 3.17; University of Manitoba, www.umanitoba.ca/chemistry/nmr/spinworks). Final plots of all graphical data were prepared with Adobe Illustrator (v. CS5).

In-house software. Instrument control and data acquisition for the nanosecond laser system were achieved using custom software written in python (python.org) using assorted libraries packaged together by the Python(x,y) project (http://code.google.com/p/pythonxy/, v. 2.6.5.6).

The software has two main programs, the kinetics mode and full spectrum mode. These differ in the method of detection and the information obtained. The full spectrum program, ccd_main_program.py, initializes the HORIBA Jobin Yvon Triax 320 spectrometer for use with the Andor iCCD camera. The timing of the camera is controlled by a Stanford Research Systems delay, controlled by GPIB serial interface. Data are read from the iCCD using the supplied dynamic link library after the desired number of exposures and displayed on screen. It can then be stored to disk.

The kinetics mode program, kinetics_main_program.py, initializes the spectrometer for use with a Hamamatsu PMT. The signal channel from the PMT is connected to the LeCroy oscilloscope equipped with GPIB interface. After the signal is found manually on the scope, the software can be used to automate the acquisition of a series of averages. The data are then displayed on screen, and can be saved to disk. The code base for the software is included as a compressed archive available as supplementary supporting information.

Calculations and propagation of uncertainty. Eq. 1 (see manuscript text) describes the calculation of the rate constant for radical-induced oxidation of the protein subunits. For clarity, the standard equations[3] used to propagate the instrumental error associated with measurement are provided below.

The error for a single rate constant measurement was calculated as a relative error, as follows:

$$E_x = f_\tau \times k_x \quad \text{where } f_\tau = \frac{E_\tau}{\tau} \tag{S2}$$

[2] Strohalm, M.; Kavan, D.; Novák, P.; Volný, M.; Havlíček, V. *Anal. Chem.* **2010**, *82*, 4648–4651.

[3] Harris, D. *Quantitative Chemical Analysis*; 6th ed. W.H. Freeman and Co.: New York, **2003**.

where E_x denotes the error for each rate constant (either E_{on} or E_{off}), f_τ is the error fraction, E_τ is the error in the lifetime measurement, and τ is the measured lifetime (τ_{on} or τ_{off}). The error for the calculated oxidation rate constant (given by equation 1), is then calculated by addition in quadrature, as follows:

$$E_t = \sqrt{{E_{on}}^2 + {E_{off}}^2} \tag{S3}$$

where E_t is the total error of the measurement, and E_{on} and E_{off} are the error of each kinetic measurement, respectively. Similarly, the error for a single lifetime measurement was propagated to an averaged lifetime measurement by averaging in quadrature.

Calculation of Radical Yield. The photochemical yield of trifluorotyrosyl radical ($\phi_{\bullet Y}$), measured in molecules of radical generated per photon, was calculated using the equation:

$$\Phi_{\bullet Y} = \frac{N_A V_{\text{Bore}} \left(\frac{\Delta OD}{\varepsilon_{\bullet Y} \ell} \right)}{\left(\frac{E_P \lambda}{hc} \right) (1 - 10^{-\varepsilon_{355,\text{Re}}[\text{Re}]\ell})} \tag{S4}$$

where N_A is avogadro's number, V_{Bore} is the volume of the cuvette in the path length (70 μL), ΔOD is the transient absorption of the radical (5.2×10^{-3}), $\varepsilon_{\bullet Y}$ is the approximate extinction coefficient of the radical itself (2,750 M^{-1} cm^{-1} for phenoxy radical),[4] ℓ is the sample path length (1 cm), E_P is the energy of each laser pulse (2.0 mJ), λ is the wavelength of observation (355 nm), h is planck's constant, c is the speed of light, $\varepsilon_{355,\text{Re}}$ is the extinction coefficient of the rhenium complex (5,300 M^{-1} cm^{-1}),[5] and [Re] is the concentration of the complex (50 μM). For the trifluorotyrosyl radical measured in the presence of Y731F–α2, $\Phi_{\bullet Y}$ = 0.049, or 4.9%.

Procedures

Tyrosine phenol lyase. TPL expression and purification was adapted from existing procedures,[6,7] with minimal changes. *E. Coli* SVS pTZTPL was available as a glycerol stock solution from previous expression in our group. A small amount (~0.5 mL) from a frozen

[4] Feitelson, J.; Hayon, E. *J Phys Chem* **1973**, *77*, 10-15.

[5] Reece, S. Y.; Seyedsayamdost, M. R.; Stubbe, J.; Nocera, D. G. *J. Am. Chem. Soc.* **2007**, *129*, 13828-13830.

[6] Chen, H.; Gollnick, P.; Phillips, R. S. *Eur. J. Biochem.* **1995**, *229*, 540–549.

[7] Kim, K.; Cole, P. A. *J. Am. Chem. Soc.* **1998**, *120*, 6851–6858.

glycerol stock was used to inoculate 2 L of LB medium containing amp (100 μg/mL), and the culture was grown at 37 °C for 20 h in an incubator at 220 RPM. Cells were harvested by centrifugation (10 min, 3,000 × *g*). The resulting cell pellet (8 g) was suspended in 35 mL of standard buffer: 100 mM KPi (pH 7.0) with 0.1 mM PLP, 1 mM EDTA, and 5 mM β-meraptoethanol. The cells were lysed by passage through a SLM-Aminco French pressure cell between 16,000 and 18,000 psi. The resulting solution was centrifuged (30 min, 25,000 × *g*) at 4 °C, and the supernatant was isolated and treated with 0.2 vol (4 mL) of 6% w/v protamine sulfate. The solution clouded immediately, and it was stirred 20 minutes more at 4 °C to ensure complete DNA precipitation. It was then centrifuged (30 min, 25,000 × *g*) at 4 °C. The supernatant was isolated. To the clear yellow solution was added solid $(NH_4)_2SO_4$ to reach 66% of saturation (39 g / 100 mL). The solution clouded immediately. It was stirred an additional 30 min at 4 °C to dissolve the salts, and it was then centrifuged (30 min, 25,000 × *g*) to pellet the protein. The supernatant was discarded, and the pellet was gently redissolved with 20 mL of standard buffer. The solution was then dialyzed in a 10 kD MWCO membrane (Pierce Biotechnology) for 18 h against 2 L of standard buffer that was 25% saturated with $(NH_4)_2SO_4$. The dialyzed protein solution was then loaded onto an octyl sepharose column, which was equilibrated with standard buffer that was 25% saturated with $(NH_4)_2SO_4$. The protein was then eluted with the same buffer and collected in 2 min fractions. The protein eluted within the first 40 fractions, as determined by TPL activity assays. The fractions containing enzyme activity were pooled and concentrated to 120 μM with a specific activity of 0.6 U/mg, flash frozen at 77 K, and stored at –80 °C. Yield 4 mL of 72 U/mL.

(*N*-Fmoc)-2,3,6-trifluorotyrosine. The fmoc protection of 2,3,6-trifluorotyrosine has been previously described.[8] It is detailed here due to minor changes in the purification that were found to increase material recovery yields. A 25 mL round bottom flask was charged with a magnetic stir bar and the fluorotyrosine (1 equiv). To this was added water (to make 0.67 M fluorotyrosine) and K_2CO_3 (10% w/v). The solution was stirred magnetically, and it became tan-brown and homogenous. The flask was then cooled with stirring in an ice bath for 10 min. During this time, Fmoc-Succinimide (1.2 equiv) was dissolved in 1,4-dioxane (0.8 M Fmoc-Suc). The dioxane solution was then added to the cold tyrosine solution dropwise over 4-5 min, giving final solution concentrations of 0.33 M fluorotyrosine and 0.4 M Fmoc-Suc. The solution was stirred while the ice melted to rt (4 h). The reaction

[8] Seyedsayamdost, M. R.; Reece, S. Y.; Nocera, D. G.; Stubbe, J. *J. Am. Chem. Soc.* **2006**, *128*, 1569–1579.

progress was monitored by TLC in 20% v/v ethyl acetate/dichloromethane containing 0.1% v/v TFA. After the reaction finished progressing, it was transferred to a separatory funnel and washed once with ethyl acetate. The solution was then acidified with ~5 mL of 1 M HCl, during which the solution became cloudy. The material was then extracted with three fractions of ethyl acetate, dried over sodium sulfate, vacuum filtered through a 1 cm pad of celite, and condensed under reduced pressure. The product was purified by flash column chromatography on silica gel. The yellow oil was dissolved in 5% v/v ethyl acetate/dichloromethane containing 0.05% v/v TFA, which was loaded onto the silica column. The product was then eluted with 20% EtOAc/DCM + 0.05% TFA, and collected in 10 mL fractions. The solution was condensed under reduced pressure, and the yellow oil was triturated with DCM, which caused the off-white product to precipitate. The suspension was then reconcentrated under reduced pressure. The trituration/evaporation procedure was repeated four additional times to ensure removal of free TFA, and the final material was dried at room temperature for 12 h at 50 mTorr. Yield 270 mg (28%), off-white solid. All characterization of the compound matched that previously reported.[8]

Table S1. Time constants (μs) and amplitudes (%) of •F_3Y TA kinetic decays. Monoexponential or biexponential weighted least-squares regression was used. The signals presented are either [Re]–F_3Y–βC19 alone or in the presence of each α2 subunit used in this study.

	λ_{det} = 395		λ_{det} = 410		λ_{det} = 425	
	τ_1	τ_2	τ_1	τ_2	τ_1	τ_2
peptide	14.7 ± 0.2	–	13.5 ± 0.2	–	14.5 ± 0.2	–
Y731F-α2	16.5 ± 0.4	–	14.2 ± 0.2	–	13.3 ± 0.2	–
Y730F-α2	14.4 ± 0.2	–	13.7 ± 0.2	–	14.0 ± 0.2	–
C439S-α2[a]	17.3 ± 1.0	2.2 ± 1.4	16.1 ± 0.3	1.3 ± 0.5	17.2 ± 0.6	3.8 ± 1.0
wt-α2[a]	15.0 ± 0.6	2.8 ± 1.0	15.1 ± 0.4	2.4 ± 1.0	16.1 ± 0.6	3.6 ± 1.8
	A_1	A_2	A_1	A_2	A_1	A_2
C439S-α2 [b]	81 ± 2	19 ± 4	81 ± 2	19 ± 4	82 ± 1	18 ± 3
wt [b]	81 ± 2	19 ± 4	85 ± 2	15 ± 4	88 ± 2	12 ± 4

[a]Biexponential decay kinetics. [b]Amplitude of the decay signal possessing corresponding lifetime given in the above header.

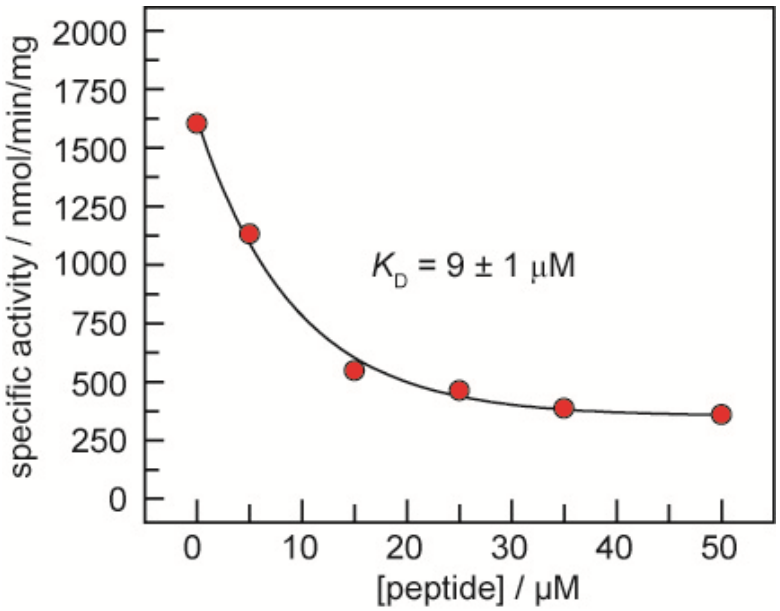

Figure S1. Competitive inhibition assay between [Re]–F_3Y–βC19 and wt RNR. The photoRNR peptide was titrated against a solution of intact wt RNR including substrate (CDP) and effector (ATP). Activity was measured using a spectrophotometric readout of NADPH consumption. For each measurement, the solution contained 100 nM wt-α2, 200 nM wt-β2, 30 μM TR, 0.5 μM TRR, 200 μM NADPH, 1 mM CDP, 3 mM ATP, 15 mM $MgSO_4$, 5% v/v glycerol and the specified amount of [Re]–F_3Y–βC19 in 50 mM borate buffer (pH 8.3).

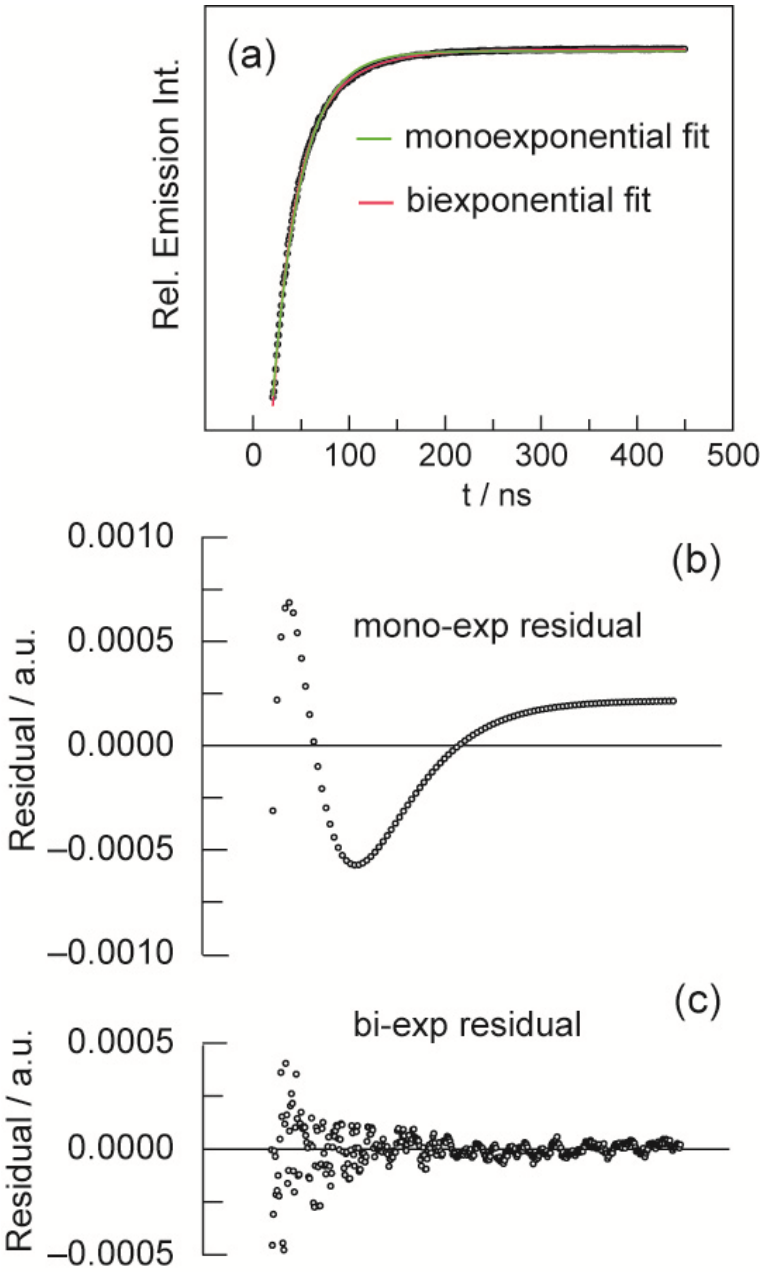

Figure S2. Time-resolved decay of emission of [Re]-F_3Y-βC19. Data were recorded at 610 nm after 7 ns 355 nm excitation over a 500 ns window (a) and fit with a monoexponential least-squares regression (b). The prominent deviation from a linear relationship between the residuals and the time variable lead us to perform a biexponential regression (c), revealing τ_1 = 58 ± 2 ns and τ_2 = 22 ± 2 ns. The solution contained 10 μM [Re]-F_3Y-βC19, 1 mM CDP, 3 mM ATP, 15 mM $MgSO_4$ and 5% v/v glycerol in 50 mM borate buffer (pH 8.3).

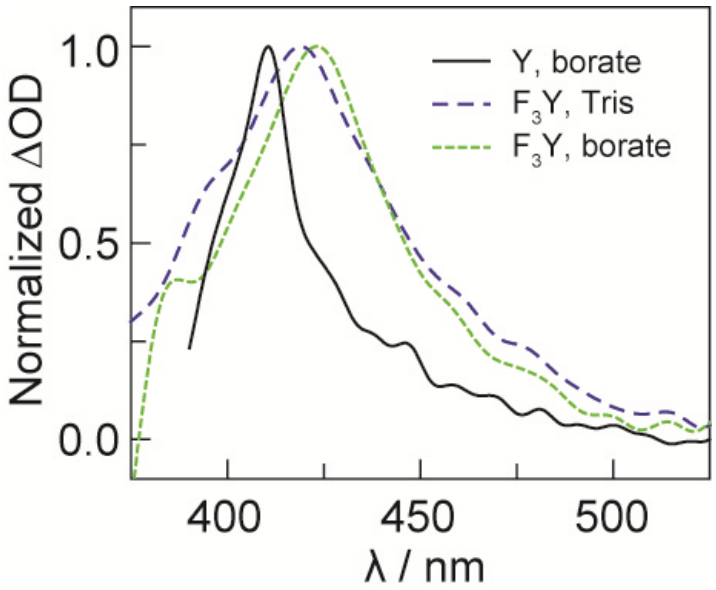

Figure S3. Normalized TA spectra of flash/quenched peptides. Data were gated over 25 ns after a 50 ns delay from the 7 ns 355 nm excitation pulse. For each measurement, a series of 500 spectra were collected using the pump/probe sequence described in equation S1, and averaged to create a single spectrum. Each final spectrum plotted here is an average of data from three samples of identically prepared solution. Individual solutions contained 100 μM [Re]–X–βC19, 200 equiv (20 mM) $Ru(NH_3)_6Cl_3$, 1 mM CDP, 3 mM ATP, 15 mM $MgSO_4$ and 5% v/v glycerol in 50 mM pH 8.3 Tris or borate buffer, as shown.

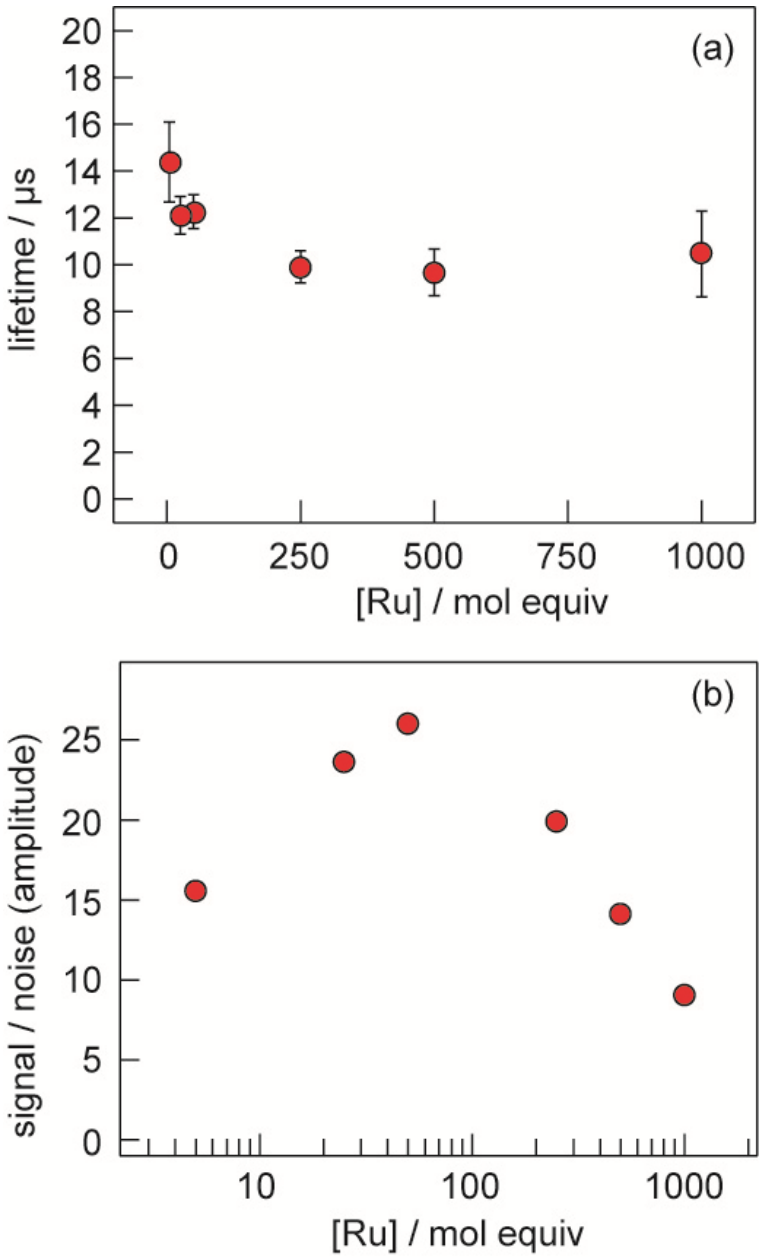

Figure S4. Optimization of flash/quench conditions for maximum •F_3Y signal intensity and lifetime. Decay of the [Re]–•F_3Y–βC19 was collected at λ_{det} = 425 nm following a 7 ns 355 nm excitation. Each decay is an average of the data collected for three samples of identically prepared solution. Each lifetime (**a**) was determined by monoexponential weighted non-linear least squares regression of the averaged data. Each solution consisted of 50 µM [Re]–F_3Y–βC19 with varying concentrations of $Ru(NH_3)_6Cl_3$ in the presence of 15 mM $MgSO_4$ and 5% glycerol in 50 mM borate buffer (pH 8.3). S/N ratios were calculated from the decays by dividing the amplitude at t = 1 µs (signal) by the amplitude between the highest and lowest data points recorded in the last 5 µs of data (noise).

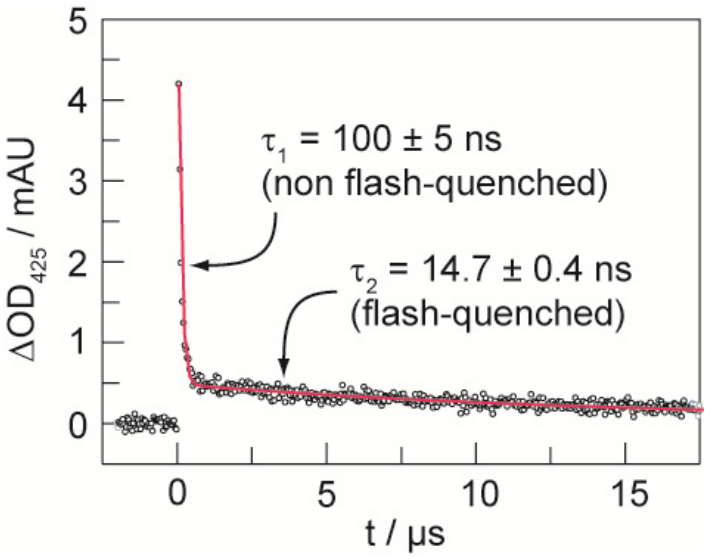

Figure S5. Decay profile at 425 nm for photoexcited [Re]–F_3Y–βC19 in optimized flash/quench conditions. Data were recorded after 7 ns 355 nm excitation of a solution containing 50 μM [Re]–[F_3Y]–βC19, 20 equiv (1 mM) $Ru(NH_3)_6Cl_3$, 1 mM CDP, 3 mM ATP, 15 mM $MgSO_4$, and 5% v/v glycerol in 50 mM borate buffer (pH 8.3). The rapid decay corresponds to [Re($bpy^{\bullet-}$)]–•F_3Y–βC19, which has not been flash-quenched; the long decay corresponds to [Re]–•F_3Y–βC19, in which the [Re] photoproduct has been flash quenched.

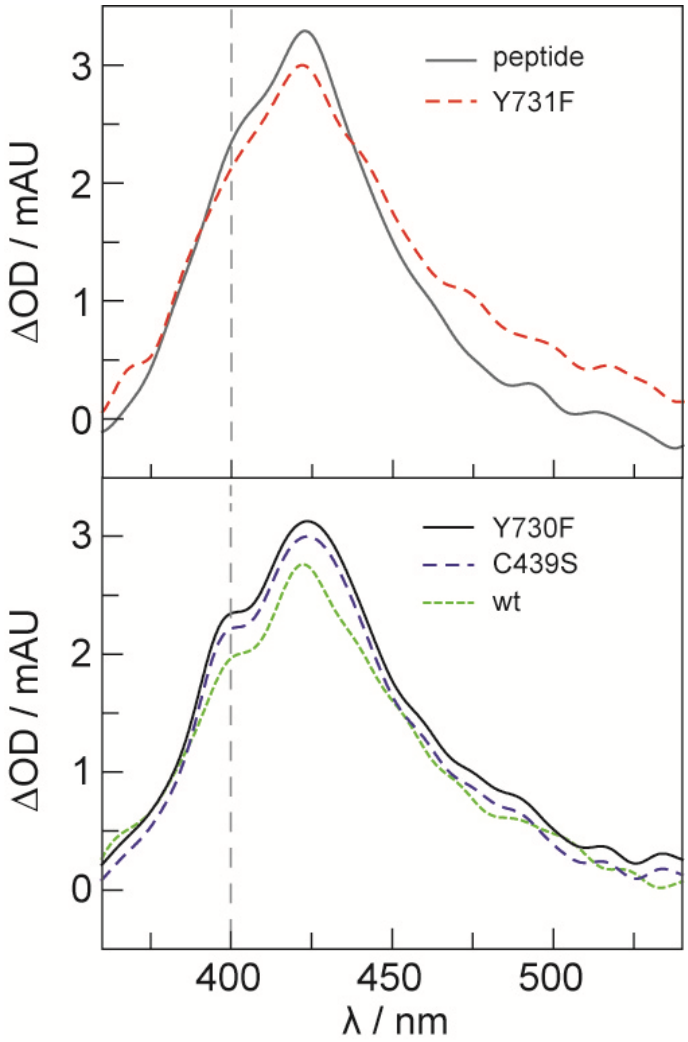

Figure S6. Spectra of photoexcited [Re]–F_3Y–βC19 alone, and in the presence of α2 variants. Data were gated over 25 ns after a 1 μs delay from the 7 ns 355 nm excitation pulse. For each measurement, a series of 500 spectra were collected using the pump/probe sequence described in equation S1, and averaged to create a single spectrum. Each final spectrum plotted here is an average of data from three samples of identically prepared solution. Each solution contained 50 μM [Re]–•F_3Y–βC19, 20 mol equiv (1 mM) $Ru(NH_3)_6Cl_3$, 1 mM CDP, 3 mM ATP, 15 mM $MgSO_4$ and 5% v/v glycerol in 50 mM borate buffer (pH 8.3).

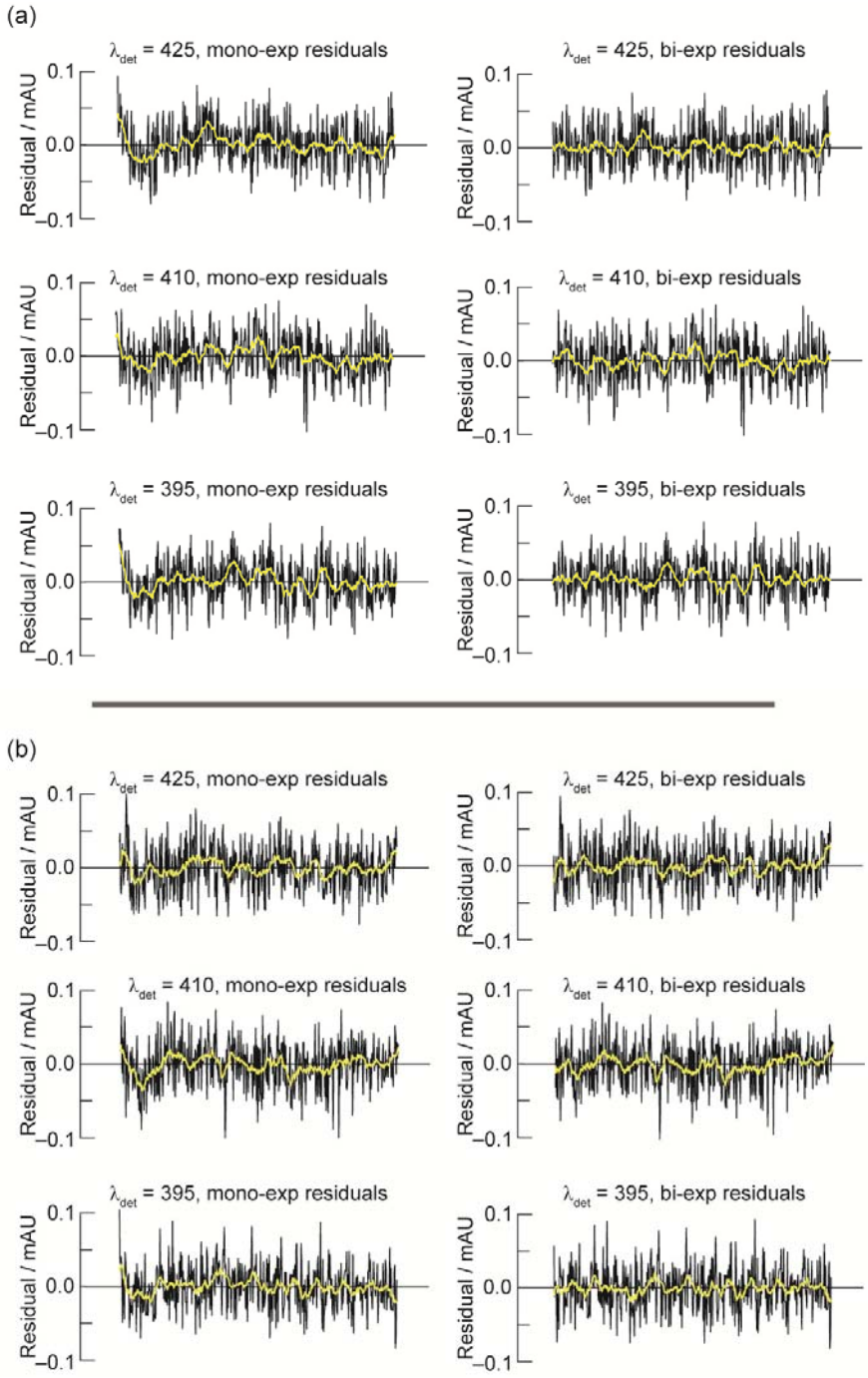

Figure S7. Graphical residual analysis of kinetic decays of [Re]–•F_3Y-βC19:C439S-α2 and Re–•F_3Y–βC19:wt-α2. (a) Monoexponential (left) and biexponential (right) residuals for [Re]–F_3Y–βC19:C439S-α2. (b) Monoexponential (left) and biexponential (right) residuals for [Re]–F_3Y–βC19:wt-α2. The trend lines are a 20-point adjacent-averaged smoothing that are included as a guide for the eye. The region that deviates from linearity is in the left half of each monoexponential trace, corresponding to a short lifetime component.

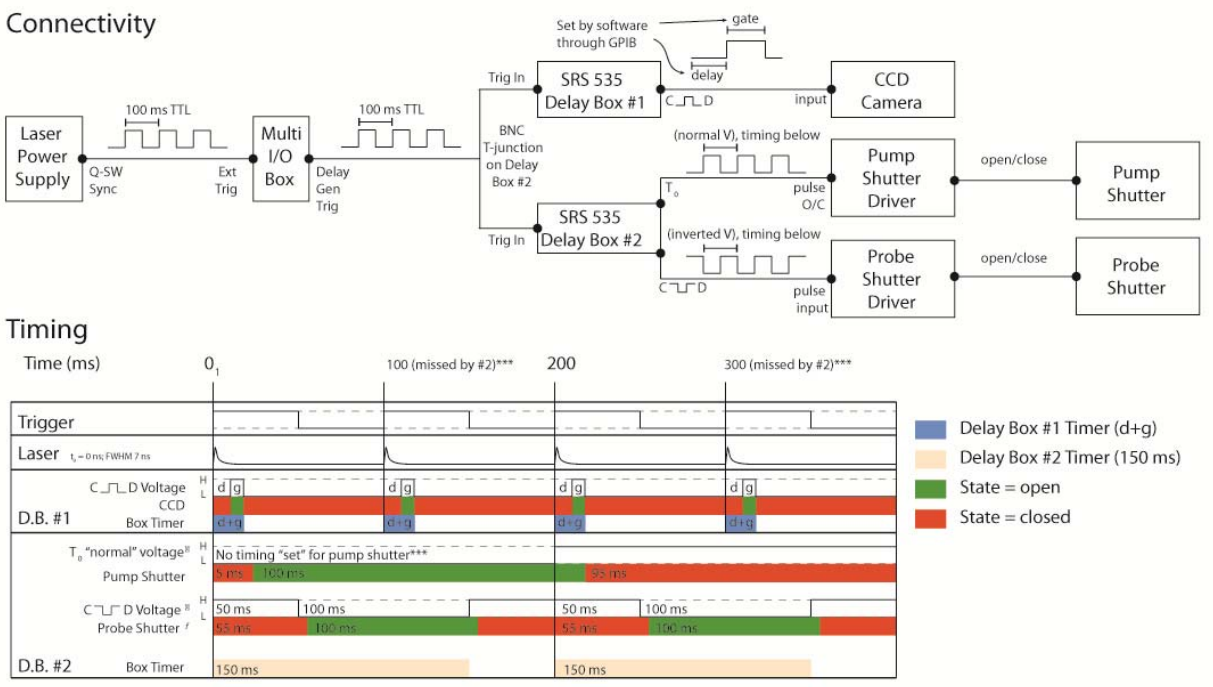

Figure S8. Connectivity and sync timing of the ns TA system. The t = 0 point is set by a TTL voltage line synced to the Q-switch (Q-SW) of the laser power supply. This signal is used to set the delay and gate of the CCD detector through delay box #1 with timings set on the fly by home-written software that commands the SRS box through a GPIB interface. The TTL signal is split, and also passed to delay box #2, which is used to control both the pump and probe shutters through hardware settings in the box. Because the shutters have a mechanical delay on the order of milliseconds, the hardware timings are set so that the opening and closing of shutters occurs as far as possible from the time points at which light will be present. The full shutter sequence is a 400 ms repeat synced to the 100 ms trigger of the Q-SW signal. It is important to note that delay box 2 only sees every second TTL signal from the Q-SW. This is by design in order to allow the full pump/probe open/closed sequence to function. The CCD, however, does receive all of the Q-SW signals so that it may record the four states listed in Eq. S1. Pump and probe shutter "open" and "closed" states are shown in the diagram in green and red, respectively. Delay and gate timings are not to scale, as they vary with collection settings.