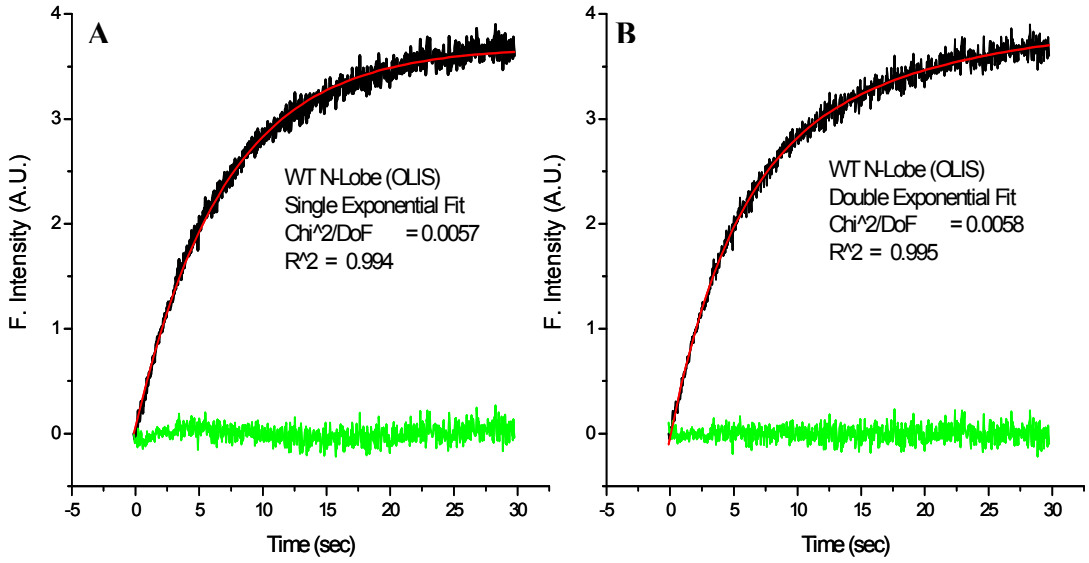
Supplemental Table 1. Fluorescence properties of hTF N-lobe and various mutants at 280 nm excitation

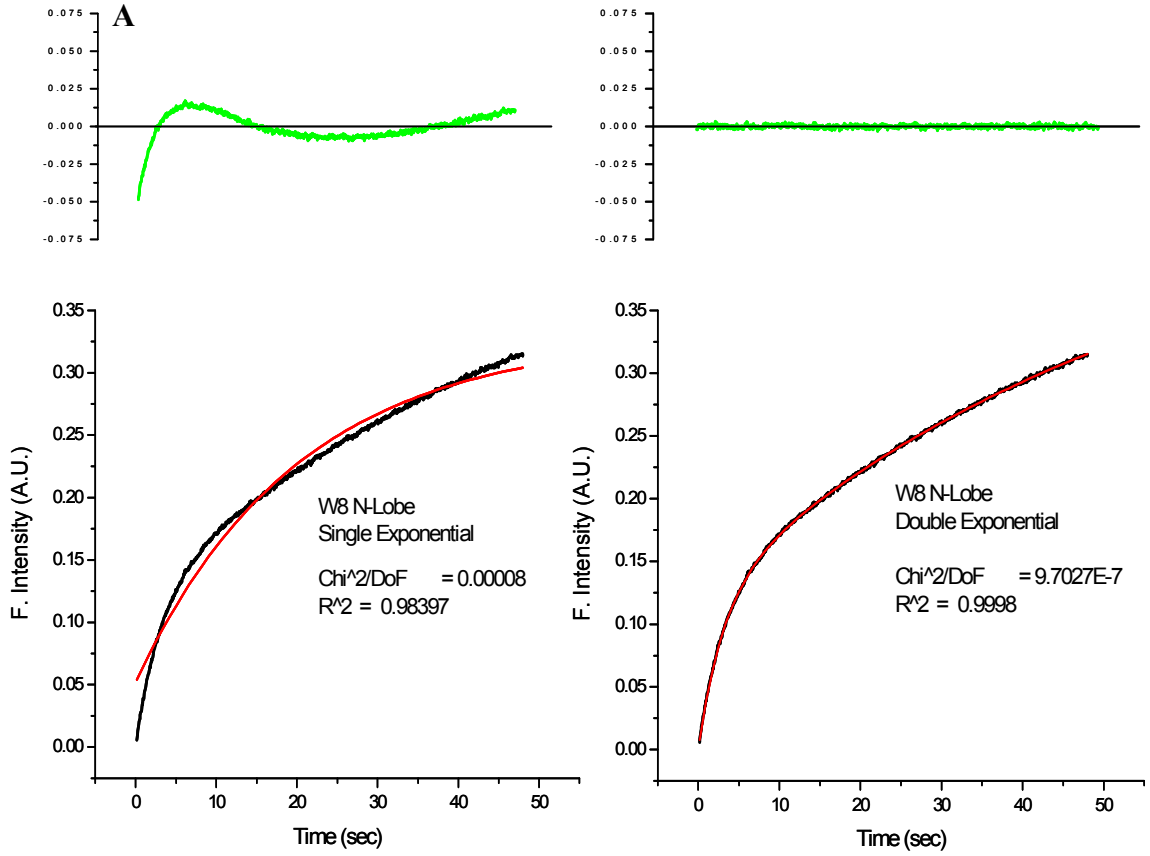
N-lobe	Relative Fluorescence Intensity at λ_{max} Counts[a]	λ_{max} (nm)
WT (Apo)	274000	338
WT (Iron)	89000	341
Trp8 (Apo)	93000 (105000 @60 min)	335
Trp8 (Iron)	70000 (89000 @10 min.)	338
Trp128 (Apo)	176000	335
Trp128 (Iron)	88000	340
Trp264 (Apo)	232000	341
Trp264 (Iron)	150000	341
Null Trp (Apo)	120000	340
Null Trp (Iron)	105000	340

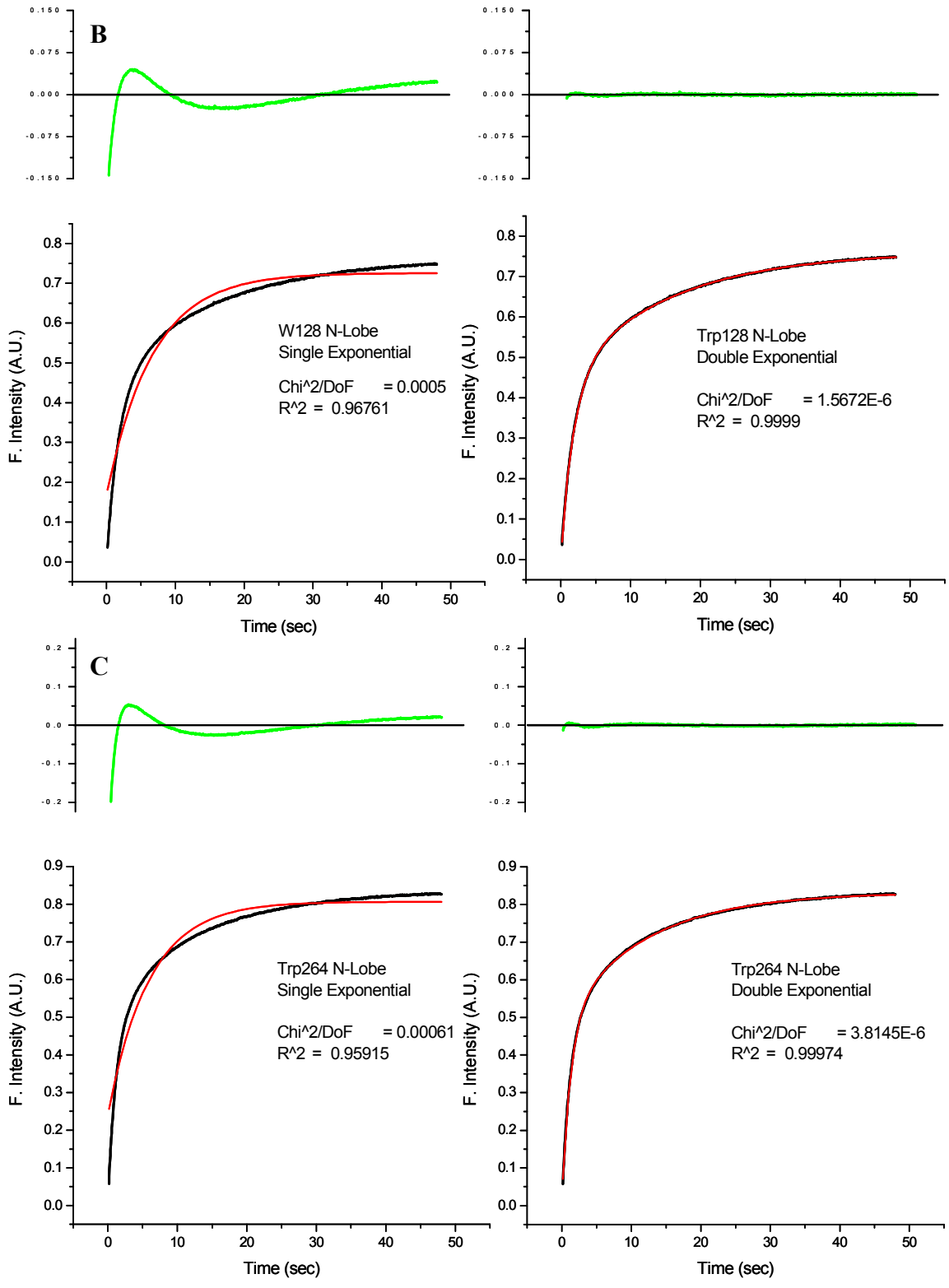
[a]Samples contained 1 μM protein. The scans of the iron containing samples are in HEPES buffer and those of the apo-samples in MES buffer as described in Methods.

Supplemental Figure 1. Iron release from WT N-lobe (6.25 μM) at pH 5.6 performed on an OLIS-RSM 1000 (Excitation slit 1.25 mm, Emission slit 3.16 mm). Iron-bound samples were rapidly mixed with MES buffer, pH 5.6 and EDTA and excited at 280 nm. The data for each curve was fit to a single exponential (**A**) and a double exponential function (**B**). The R^2, Chi^2/DoF, and residuals (green) are provided on the figures and clearly show no significant difference between the single and double exponential fits

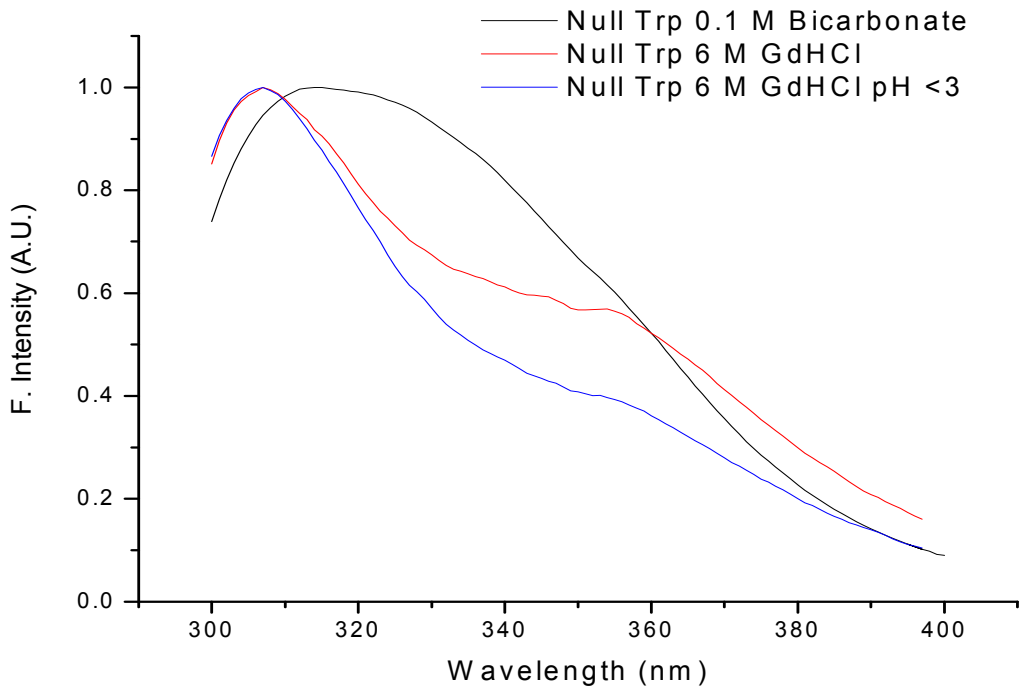


Supplemental Figure 2. Iron release from double Trp N-lobe mutants at pH 5.6 performed on an Applied Photophysics stopped-flow spectrofluorometer. Iron-bound samples were rapidly mixed with MES buffer, pH 5.6 and EDTA and excited at 280 nm. The data for each curve was fit to a single exponential or a double exponential function to justify the selection of the two-exponential fit for each of the mutants: (**A**) Trp8; (**B**) Trp128; and (**C**) Trp264 N-Lobe. The R^2, Chi^2/DoF, and residuals (in green) are provided on the figures.




B
W128 N-Lobe
Single Exponential
Chi^2/DoF = 0.0005
R^2 = 0.96761
Trp128 N-Lobe
Double Exponential
Chi^2/DoF = 1.5672E-6
R^2 = 0.9999
C
Trp264 N-Lobe
Single Exponential
Chi^2/DoF = 0.00061
R^2 = 0.95915
Trp264 N-Lobe
Double Exponential
Chi^2/DoF = 3.8145E-6
R^2 = 0.99974
F. Intensity (A.U.)
Time (sec)

Supplemental Figure 3. Normalized steady-state emission scans of the null Trp mutant in 100 mM ammonium bicarbonate, pH ~8 or in 6 M GdHCl, pre and post acidification of the sample. Excitation wavelength was set at 280 nm for maximal excitation of tyrosine residues.

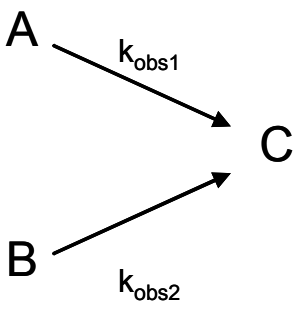


APPENDIX

Biphasic Kinetic Modeling

Kinetic data obtained by monitoring intrinsic Trp fluorescence as a result of iron release from the isolated N-lobe of hTF at pH 5.6 required a double exponential fit. These results differ from our previous studies *(1)*. A detailed kinetic model that fits our data is presented below. Given the double exponential fit, four kinetic models have been considered and are described below along with their respective equations.

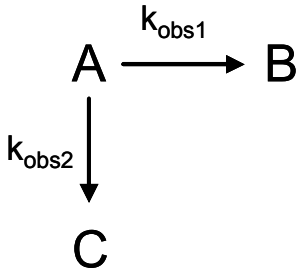
1.



$$[A] = [A_0] * e^{-kobs1t}$$

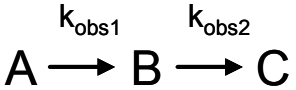
$$[B] = [B_0] * e^{-kobs2t}$$

2.



$$[A] = [A_0] * (e^{-kobs1t} + e^{-kobs2t})$$

3.

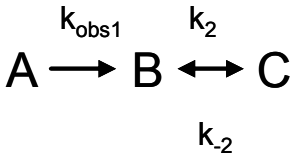


$$[A] = [A_0] * e^{-kobs1t}$$

$$[B] = k_1[A_0] * ([1/(k_2 - k_1)]*e^{-k1t} + [1/(k_1 - k_2)]*e^{-k2t})$$

$$[C] = [A_0] (1- k_2/(k_1 - k_2)*e^{-k1t} - k_1/(k_2 - k_1)*e^{-k2t})$$

4.



$$[A] = [A_0] * e^{-kobs1t}$$

$$[B] = k_1*[A_0] * ((k_{-2}/(\gamma_1\gamma_2) - (k_{-2} - \gamma_1)/(\gamma_1(\gamma_2 - \gamma_1)*e^{-\gamma 1t} - ((k_{-2} - \gamma_2)/(\gamma_2(\gamma_1 - \gamma_2)))*e^{-\gamma 2t})$$

$$[C] = (k_1*(k_{-2} - k_1) * [A_0]) *(1/(\gamma_1\gamma_2) - 1/(\gamma_1(\gamma_2 - \gamma_1)) *e^{-\gamma 1t} - 1/(\gamma_2(\gamma_1-\gamma_2))*e^{-\gamma 2t})$$

where $\gamma_1 = k_{obs1}$ and $\gamma_2 = k_{obs2} = k_2 + k_{-2}$

To definitively assign one of the observed rates from the fluorescence data to iron release, we set up the stopped-flow instrument to monitor the decrease in absorbance at 470 nm. Because the released iron is bound by a chelator, step 1 is by definition irreversible. This visible absorption band is a product of ligand to metal charge transfer and is only present when iron is coordinated by the two tyrosine ligands *(2)*. The data fit best to a single exponential with an observed rate constant equal to that of the faster rate obtained in the fluorescence studies. If either Model 1 or 2 was correct then monitoring the decrease of iron bound N-lobe (*i.e.*, loss of [A] and [B] for Model 1 or loss of [A] for Model 2) would have yielded biphasic kinetics with rate constants identical to those obtained in the fluorescence work. This was not the case and therefore Models 1 and 2 can be eliminated. Both Models 3 and 4 are consistent with our data. In order to determine the correct model, our fluorescence data was fit to each set of equations. The fit from Model 4 provided a k_{-2} rate that was very small (0.00005 sec^{-1}). This indicates that the reaction is driven to the right and the reverse reaction is virtually non existent, making this model effectively Model 3

In Model 3 A is defined as iron bound N-lobe in the closed conformation, B is apo-N-lobe in the closed conformation and C is apo-N-lobe in the full open state. Model

3 specifies that when iron is released A goes to B in an irreversible manner, which should result in an enhancement in fluorescence. After iron is released the N-lobe undergoes a slower conformational event (B going to C) that perturbs the local environment around two of the three Trp residues, also resulting in an increase in fluorescence. Based on this model, $k_{obs1} = k_1$ and $k_{obs2} = k_2$ for the derivations below

$$A \xrightarrow{k_{obs1}} B \xrightarrow{k_{obs2}} C$$

$$d[A]/dt = -k_1[A] \quad \text{(Eq. 1)}$$

The term d/dt is replaced with the LaPlace operator p and because [A] is $\neq 0$ at t = 0, we must consider the initial concentration of A, A_0, and subtract d[A]/dt by $d[A_0]/dt$:

$$p[A] - p[A_0] = -k_1[A] \quad \text{(Eq. 2)}$$

Rearrange Eq. 2 and combine like terms

$$(p + k_1)[A] = p[A_0] \quad \text{(Eq. 3)}$$

Divide each side by $(p + k_1)$:

$$[A] = p[A_0]/(p + k_1) \quad \text{(Eq. 4)}$$

Replace the equation with the correct inverse transformation taken from the LaPlace Transformation table *(3)*:

$$[A] = [A_0]e^{-k1t} \quad \text{(Eq. 5)}$$

Next we have to analyze [B], since $[B_0] = 0$ at t = 0, we do not consider a B_0 term.

$$d[B]/dt = k_1[A] - k_2[B] \quad \text{(Eq. 6)}$$

LaPlace transformation:

$$p[B] + k_2[B] = k_1[A] \quad \text{(Eq. 7)}$$

Rearrange and combine like terms:

$(p + k_2)[B] = k_1[A]$ (Eq. 8)

Replace [A] with Eq. 4 and divide through by $(p + k_2)$:

$[B] = k_1[A_0]p/((p + k_1)(p + k_2))$ (Eq. 9)

Replace the equation with the correct inverse transformation taken from the table *(3)*

$[B] = k_1[A_0] * ([1/(k_2 – k_1)]*e^{-k1t} + [1/(k_1 – k_2)]*e^{-k2t})$ (Eq. 10)

Lastly, we must consider C:

$d[C]/dt = k_2[B]$ (Eq. 11)

The law of mass action states that $[A_0] = [A] + [B] + [C]$, which can be rearranged to [B] $= [A_0] – [A] – [C]$.

$d[C]/dt = k_2[A_0] – k_2[A] – k_2[C]$ (Eq. 12)

Rearrange, combine like terms and replace [A] with Eq. 4

$(p + k_2)[C] = k_2[A_0] – k_2[A_0]p/(p + k_1)$ (Eq. 13)

Multiply both sides by $(p + k_1)$

$(p + k_1)(p + k_2)[C] = pk_2[A_0] + k_1k_2[A_0] - k_2[A_0]p$ (Eq. 14)

Pull out $k_2[A_0]$ from each term on the right side equation

$(p + k_1)(p + k_2)[C] = k_2[A_0](p + k_1 – p)$ (p's cancel out) (Eq. 15)

$(p + k_1)(p + k_2)[C] = k_1k_2[A_0]$ (Eq. 16)

Divide both sides by $(p + k_1)(p + k_2)$

$[C] = k_1k_2[A_0]/((p + k_1)(p + k_2))$ (Eq. 17)

LaPlace Transformation from table *(3)*:

$[C] = [A_0] (1- k_2/(k_1 – k_2)e^{-k1t} – k_1/(k_2 – k_1)e^{-k2t})$ (Eq. 18)

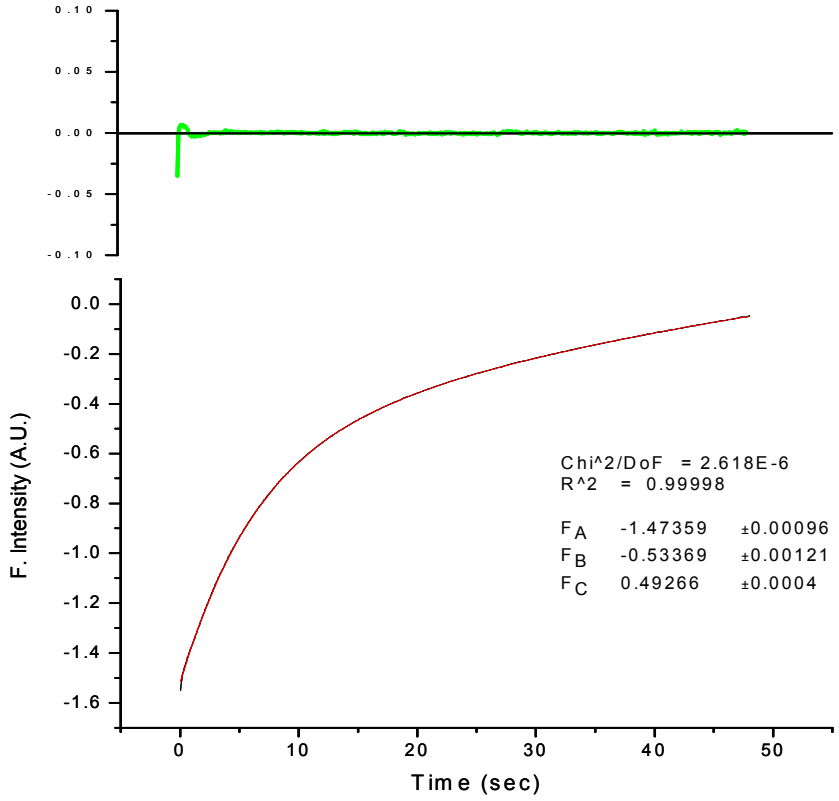
Eq. 5, 10, and 18 can be used to provide information on the actual fluorescence maximum of each of A, B and C due to the relationship between the concentration of each species and the fractional fluorescence. The fluorescence total (F_T) is equal to:

$$F_T(t) = f_A*F_A + f_B*F_B + f_C*F_C = [A]/[A_0]*F_A + [B]/[A_0]*F_B + [C]/[A_0]*F_C \quad \text{(Eq. 19)}$$

where f_A, f_B and f_C are the fractional concentrations of A, B and C and F_A, F_B and F_C are the fluorescence intensities produced by each of the states. The equation then simplifies to become:

$$f_A*F_A + f_B*F_B + f_C*F_C = (F_A + (F_B * k_1 + F_C * k_2)/(k_2 - k_1)) * e^{-k1t} + ((k_1*(F_B + F_C))/(k_1 - k_2))*e^{-k2t} + F_C \quad \text{(Eq. 20)}$$

We then fit the raw data to Eq. 20 to solve for the fluorescent state of each species, by fixing k_1 and k_2 to the rates obtained from the original double exponential fit:



Based on the fit, the fluorescent state of closed, apo-N-lobe (B) is ~ 80% greater than Fe-N-lobe (A) and the final state, apo-N-lobe (C) is ~ 170% greater than A. This overall change is further confirmed by our steady-state data, which shows that the fully open, apo-form of the hTF N-lobe has a fluorescent signal that is ~ 230% higher than the iron bound form. This correlates with the observed increase by stopped-flow fluorescence of 170% from A→ C. The ability to obtain the same values for the fluorescence contribution of apo-N-lobe "open" using two independent experiments is proof of principle.

References:

1. Mason, A. B., Halbrooks, P. J., James, N. G., Connolly, S. A., Larouche, J. R., Smith, V. C., MacGillivray, R. T. A., and Chasteen, N. D. (2005) Mutational analysis of C-lobe ligands of human serum transferrin:insights into the mechanism of iron release, *Biochemistry 44*, 8013-8021.

2. Patch, M. G. and Carrano, C. J. (1981) The origin of the visible absorption in metal transferrins, *Inorg. Chim. Acta 56*, L71-L73.

3. Rodigin, N. M. and Rodigina, E. M. (1964) Consecutive Chemical Reactions: Mathematical Analysis and Development, 1st edition. Princeton, N.J., Van Nostrand