Distance Mapping in Proteins Using Fluorescence Spectroscopy:

The Tryptophan-Induced Quenching (TrIQ) Method

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Supporting Information

Analysis method for determining the relative fraction of static and dynamic quenching in a TrIQ Study.

I. Overview.

In a TrIQ study, the fraction of Trp-fluorophore that are not in a static complex (γ) and the fraction that is in a static-complex (1- γ) are determined in the following way:

Experimental:

1. Measure (F_w/F_0), the total fluorescence intensity of the samples with (F_w) and without (F_0) the quenching Trp residue.
2. Measure (τ_w/τ_0), the fluorescence lifetime of the sample with (τ_w) and without (τ_0) the quenching Trp residue.

Analysis:

1. Calculate the relative fraction of Trp-fluorophore pairs not in a static complex using:

$$\gamma = \frac{F_w}{F_0} \cdot \frac{\tau_0}{\tau_w}$$

2. Calculate the relative fraction of Trp-fluorophore pairs involved in a static complex using (1- γ).

3. The fraction of fluorophores in γ that emit light without being quenched can be calculated as $\gamma_F = \frac{\tau_w}{\tau_0} \cdot \gamma$. Similarly, the fraction of fluorophores that undergo dynamic quenching can be calculated from $\gamma_{DQ} = \left(1 - \frac{\tau_w}{\tau_0}\right) \cdot \gamma$

The only requirement is that the quenching process follows the scheme outlined in **Figure 4** of the main text.

II. Description of the above analysis.

The relative fraction of Trp/fluorophores involved in a static, non-fluorscent complex can be calculated by adapting an analysis method previously used by Weber and colleagues to measure the amount of static and dynamic quenching occurring in FAD (*1-3*). Unfortunately, those manuscripts use somewhat confusing terminology. Thus, to ensure the applicability of this analysis approach to our TrIQ studies, we re-derived the re-confirmed the calculations for determining the amount of intramolecular static quenching. A brief synopsis is given below.

After collecting both steady-state and lifetime data, the analysis begins by first calculating γ, the relative fraction of absorption transitions that occur by free, unquenched (uncomplexed) fluorophores in the ground state, relative to the total number of absorptions by the fluorophore. This is calculated from the fluorescence data as:

$$\gamma = \left(\frac{F_w}{F_0}\right)\left(\frac{\tau_0}{\tau_w}\right)$$

where F_w represents the quantum yield (or integrated steady-state fluorescence intensity) of the fluorophore in the presence of a Trp, while F_0 represents the quantum yield (or integrated steady-state fluorescence intensity) in the absence of the Trp residue. Similarly, the values τ_w and τ_0 represent the fluorescence lifetime of the fluorophore with and without the presence of the Trp residue, respectively.

Note - in TrIQ studies, it is necessary to use the amplitude weighted fluorescence lifetime, <τ>, which is defined as: $< \tau >= \sum \alpha_i \tau_i$, where α_i is the normalized amplitude factor for each lifetime, τ_i. This is necessary because one rarely observes a single lifetime for the quenched sample in a TrIQ study – the fluorescence decay data must usually be fit using two or more lifetimes. The amplitude-weighted lifetime is the best method for this analysis, because <τ> is directly proportional to the area under the decay curve, and thus is proportional to the steady-state fluorescence intensity (*4*).

III. Theory behind calculating static and dynamic quenching in TrIQ studies.

Below we provide a brief derivation demonstrating how the relative fraction of static quenching (non-fluorescent complex formation) can be determined from fluorescent data in a TrIQ study.

In TrIQ studies, the quencher and fluorophores are linked together on the same molecule. Thus, each labeled protein contains a fluorophore-quencher pair in a 1:1 molar ratio. In the dilute solutions used in most fluorescence studies, each pair will then behave independently of concentration and the state of the other molecules in solution. This means that the tryptophan-induced quenching of fluorophores is an intramolecular phenomenon, and the quenching is <u>concentration independent</u>, which can be described as:

$$F-Q \underset{k_-}{\overset{k_+^*}{\rightleftharpoons}} FQ$$

Scheme 1

where [FQ] is the concentration of the covalently-linked fluorophore-quencher complex in a static complex, and [F-Q] represents the "free" fluorophores not in a static complex (but obviously still linked to the same molecule).

The equilibrium constant of association is then given by:

$$K_A = \frac{[FQ]}{[F-Q]}$$

Note – in the derivation below, we assume:

1. FQ is non-fluorescent.
2. F-Q is fluorescent.

3. k_+^* reflects the forward rate constant for forming a non-fluorescence FQ complex.

As is described in Fig. 4 of the main text, in a TrIQ study, a decrease in the fluorescence intensity of a population of fluorescent molecules is caused primarily by two factors:

(1) Dynamic quenching –caused by interactions of the excited fluorophore with the Trp quencher during the lifetime of the excited state. This can also also be referred to as collisional quenching.

(2) Static quenching - caused by interaction of the fluorophore with the Trp quencher in the ground state (before absorption of a photon), forming a non-fluorescent complex.

These two types of quenching are discussed in more detail below.

III.A. Dynamic quenching in TrIQ. In *intramolecular* quenching, the quencher and fluorophores are linked together on the same molecule. Thus, intramolecular dynamic quenching can be described by:

$$F - Q \underset{k_-}{\overset{k_+^*}{\rightleftharpoons}} FQ$$

Scheme 1.

When the dynamic quenching is highly-efficient, the effect of quenching on the fluorescence lifetime (τ_0) and quantum yield (F_0) is described by a modified version of the well-known Stern-Volmer quenching equation (*4*).

$$\frac{F_0}{F_w} = \frac{\tau_0}{\tau_w} = 1 + \tau_0 \cdot k_+^* \qquad (1)$$

where τ_w and F_w represent the new lifetime and quantum yield in the presence of the Trp quencher, and k_+^* reflects the dynamic quenching constant, or rate of quenching.

Inversion of this relationship produces:

$$\frac{F_w}{F_0} = \frac{\tau_w}{\tau_o} = \frac{1}{1 + \tau_o \cdot k_+^*} \quad (2)$$

Note how these equations demonstrate that for purely dynamic quenching, a change in intensity (F) should mirror a change in lifetime (τ).

III.B. Static quenching in TrIQ. The total fluorescence intensity (F_w/F_0) can also be affected by *static* quenching, which occurs when the quencher and fluorophore have formed a non-fluorescent complex in the ground-state, either before or instantaneously after absorbing the exciting photon.

Either way, because these complexes never emit light, they reduce the overall fluorescence intensity (F_w/F_0), but have no effect on the observed rate of fluorescence decay (the ratio of fluorescence lifetime, τ_w/τ_0).

The extent to which non-fluorescent, static complexes reduce the overall fluorescence intensity can be accommodated by introducing a new factor, γ, into the relationship described in Equation 2:

$$\frac{F_w}{F_o} = \gamma \cdot \frac{1}{1 + \tau_o \cdot k_+^*} \quad (3)$$

where γ is the fraction of absorption transitions by free, uncomplexed fluorophores [F] compared to the total absorption transitions ([F] + [FQ]). Thus, γ can be described as:

$$\gamma = \frac{\varepsilon_F \cdot [F]}{\varepsilon_F \cdot [F] + \varepsilon_C \cdot [FQ]} \quad (4)$$

where ε_F is the molar extinction coefficient of the free, non-complexed fluorophores [F], and ε_c is the molar extinction coefficient of the complex [FQ] between the fluorophores [F] and quencher [Q].

If either the extinction coefficient of the open and closed complex are the same (i.e., $\varepsilon_F = \varepsilon_c$), or, if the excitation is at an isosbestic wavelength, then γ can be used to describe the relative fraction of fluorescence from the probes not involved in a static complex, and thus it reflects their relative population.

Combining equations 2 and 3 yields:

$$\gamma = \frac{F_w}{F_0} \cdot \frac{\tau_0}{\tau_w} \tag{5}$$

and

$$k_+^* = \left(\frac{1}{\tau_w} - \frac{1}{\tau_0} \right) \tag{6}$$

Equation (5) shows that γ can be directly determined by simultaneously measuring the fluorescence lifetimes (τ_w and τ_0) and the quantum yields (F_w and F_0) in the presence and absence of quencher, respectively.

Once γ been calculated, it is simple to determine the degree of "static" quenching (the fraction of complexed fluorophores at the moment of photon absorption). Because γ represents the fraction of fluorophores that are free from complex at the moment of excitation, the fraction of fluorophores that are undergoing **static quenching is defined as 1-γ.**

III.C. The γ value can be further analyzed to determine the relative fraction of unquenched fluorophores and dynamically quenched fluorophores.

As defined in the preceding discussion, the value of γ represents the fraction of total fluorophores that absorb a photon from an open, noncomplexed conformation.

However, as shown in **Figure 4** of the main text, the fluorophores that comprise γ can have two fates – they can either re-emit the photon through fluorescence (ie., be unquenched) or they can lose their ability to fluorescence through a dynamic mechanism (ie., quenching that occurs during the lifetime of the excited state).

Thus, the value of γ can be thought of as being made up of a fraction of fluorophores that emit fluorescence without being quenched (γ_F) and the fraction that are dynamically quenched (γ_{DQ}), such that $\gamma = \gamma_{DQ} + \gamma_F$. This is accomplished by comparing the rate of dynamic quenching ($k_+^* = \frac{1}{\tau_w} - \frac{1}{\tau_0}$) to the rate of unquenched fluorescence ($k_f = \frac{1}{\tau_0}$), where τ_w is the fluorescence

lifetime in the presence of a quenching tryptophan and τ_0 is the fluorescence lifetime in the absence of a quenching tryptophan:

$$\gamma_{DQ} = \frac{k_+^*}{k_+^* + k_f} \cdot \gamma \tag{7}$$

$$\gamma_F = \frac{k_f}{k_f + k_+^*} \cdot \gamma \tag{8}$$

Substituting $k_+^* = \frac{1}{\tau_w} - \frac{1}{\tau_0}$ and $k_f = \frac{1}{\tau_0}$ into Equation (8) yields:

Relative fraction of unquenched fluorescence, (γ_F)

$$\gamma_F = \frac{\frac{1}{\tau_0}}{\frac{1}{\tau_0} + \left(\frac{1}{\tau_w} - \frac{1}{\tau_0}\right)} \cdot \gamma = \frac{\frac{1}{\tau_0}}{\frac{1}{\tau_w}} \cdot \gamma$$

$$\gamma_F = \frac{\tau_w}{\tau_0} \cdot \gamma \tag{9}$$

Relative fraction of dynamic quenching, (γ_{DQ}):

$$(\gamma_{DQ}) = \left(1 - \frac{\tau_w}{\tau_0}\right) \cdot \gamma \tag{10}$$

This analysis was used to calculate the relative contributions shown in **Supplemental Figure 2.**

IV. Alternative derivation for calculating the relative fraction of static-complex formation from TrIQ data:

Below we provide a slightly different derivation demonstrating that γ ρepresents the fraction of fluorophores that are in the open conformation at the time of photon absorption in a TrIQ study:

As before, the interaction of a fluorophore with a quencher can be described as:

$$F - Q \underset{k_-}{\overset{k_+}{\rightleftharpoons}} FQ$$

The association constant (K_A) for this system is:

$$K_A = \frac{[FQ]}{[F-Q]}$$

and the fraction of fluorophores that are not complexed (*f*) is given by:

$$f = \frac{[F-Q]}{[F-Q]+[FQ]}$$

Thus:

$$f = \frac{1}{1+\frac{[FQ]}{[F-Q]}}$$

$$f = \frac{1}{1+K_A} \qquad \text{(A1)}$$

Equation A1 is an expression that represents the fraction of fluorophores that are not complexed and are thus in an "open conformation".

<u>Classic Collisional (Dynamic) Quenching:</u>

For intramolecular quenching, one can measure the rate of quenching, using a modified version of the Stern-Volmer equation (*4*):

$$\frac{F_0}{F_w} = \frac{\tau_0}{\tau_w} = 1 + k_q \tau_0 \qquad \text{(A2)}$$

where τ_0 and F_0 are the fluorescence lifetime and quantum yield in the absence of quencher, τ_w and F_w are the fluorescence lifetime and quantum yield in the presence of quencher, k_q is the dynamic quenching constant. In the case of TrIQ experiments, the quencher is a tryptophan (Trp, or W) residue, and so the subscript *w* always indicates the presence of the Trp quencher.

If we make the following definitions: $D.Q. = k_q \cdot \tau_0$ and $\delta = \frac{1}{\tau_0}$ then we can re-arrange Equation A2 as follows to get:

$$\frac{F_0}{F_w} = \frac{\tau_0}{\tau_w} = \frac{\frac{1}{\tau_0} + \frac{k_q \cdot \tau_0}{\tau_0}}{\frac{1}{\tau_0}} = \frac{\delta + k_q}{\delta} = \frac{1 + \frac{k_q}{\delta}}{1} = 1 + k_q \cdot \tau_0 = 1 + D.Q. \qquad \text{(A3)}$$

We can also arrive at a similar expression considering the fraction of excited fluorophores that decay by emission, relative to the total fluorescence. This fraction ($\frac{F_w}{F_0}$) is given by the ratio of the decay rate in the absence of quencher (δ) to the total decay rate in the presence of quencher ($\delta + k_q$):

$$\frac{F_w}{F_0} = \frac{\delta}{\delta + k_q} = \frac{1}{1 + \frac{k_q}{\delta}} = \frac{1}{1 + k_q \cdot \tau_0} = \frac{1}{1 + D.Q.} \qquad \text{(A4)}$$

Equation A3 and Equation A4 are inverted forms of the same equation.

<u>Combined Dynamic and Static Quenching:</u>

In the situation where there is both dynamic quenching and static quenching, the fractional fluorescence remaining ($\frac{F_w}{F_0}$) is given by the product of the fraction of free fluorophores (those fluorophores that are

not involved in a static complex upon light absorption), (*f*), and the fraction of fluorophores that are not quenched by collisional encounters (i.e., dynamic intramolecular quenching), ($\frac{\delta}{\delta + k_q}$):

$$\frac{F_w}{F_0} = (f) \cdot \left(\frac{\delta}{\delta + k_q} \right) \qquad \text{(A5)}$$

We can invert this equation to produce:

$$\frac{F_0}{F_w} = \left(\frac{1}{f} \right) \cdot \left(\frac{\delta + k_q}{\delta} \right)$$

If we recall that $f = \frac{1}{1 + K_A}$ (from Equation 1), and $\delta = \tau_0^{-1}$ and K_A is the association constant, and we make these substitutions into the above equation, we have:

$$\frac{F_0}{F_w} = (1 + K_A) \frac{\left(\frac{1}{\tau_0} + k_q \right)}{\frac{1}{\tau_0}}$$

Multiply both sides by : $\frac{\tau_0}{\tau_0}$

$$\frac{F_0}{F_w} = (1 + K_A) \frac{\left(\frac{\tau_0}{\tau_0} + k_q \cdot \tau_0 \right)}{\frac{\tau_0}{\tau_0}}$$

$$\frac{F_0}{F_w} = (1 + K_A)(1 + k_q \cdot \tau_0)$$

$$\frac{F_0}{F_w} = (1 + K_A)(1 + D.Q.) \qquad \text{(A6)}$$

As we saw from Equation A3: $\frac{\tau_0}{\tau_w} = 1 + D.Q.$

Substituting this into Equation A6 gives:

$$\frac{F_0}{F_w} = \left(1 + K_A\right) \cdot \frac{\tau_0}{\tau_w}$$

Inverting this equation produces:

$$\frac{F_w}{F_0} = \frac{1}{1 + K_A} \cdot \frac{\tau_w}{\tau_0}$$

If we define: $\gamma = \frac{1}{1 + K_A}$

This can be written as: $\frac{F_w}{F_0} = \gamma \cdot \frac{\tau_w}{\tau_0}$

We have previously shown from Equation A1 that the value of $\frac{1}{1 + K_A}$, which we now define as γ, equals the fraction of absorption transitions by free fluorophores.

$\therefore \gamma = \frac{F_w}{F_0} \cdot \frac{\tau_0}{\tau_w}$, a value that represents the fraction of free fluorophores that absorbed a photon and have the potential to be quenched in a dynamic process.

Thus, the value of 1-γ represents the fraction of molecules that are in a ground-state, static complex.

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Supplemental Table 1: **Spectral Characteristics of Labeled T4 Lysozyme Samples.**

Mutant	mol of label/mol of T4L	Abs. λ_{max} (nm)	Emm. λ_{max} (nm)	Quantum Yield (Φ)	Steady-State Anisotropy (r)[d]
*Q123-B_1	0.9	388	468.9±0.4	0.220±0.017	ND
*N116W/Q123-B_1	1.0	390	469.1±0.8	0.135±0.004	ND
*E128-B_1	1.0	401	469.5±0.5	0.182±0.001	ND
*N116W/E128-B_1	1.0	397	469.7±0.5	0.043±0.003	ND
*N132-B_1	0.9	386	466.8±0.1	0.201±0.004	ND
*N116W/N132-B_1	0.9	394	468.9±0.4	0.047±0.004	ND
*K135-B_1	0.9	391	472.0±0.7	0.146±0.023	ND
*N116W/K135-B_1	1.0	390	471.0±0.3	0.118±0.022	ND
Q123-qBBr	0.93[a]	378.9[a]	470[a]	0.065[a]	ND
N116W/Q123-	0.98[a]	378.9[a]	469[a]	0.037[a]	ND
E128-qBBr	0.95[a]	384.8[a]	467[a]	0.093[a]	ND
N116W/E128-	0.89[a]	379.2[a]	467[a]	0.018[a]	ND
N132-qBBr	0.76[a]	375.0[a]	468[a]	0.088[a]	ND
N116W/N132-	0.97[a]	396.1[a]	467[a]	0.014[a]	ND
K135-qBBr	0.90[a]	374.2[a]	471[a]	0.061[a]	ND
N116W/K135-	1.02[a]	374.5[a]	471[a]	0.048[a]	ND
Q123-BDPY	0.9[a]	508.1±0.1[c]	529.1±0.2[b]	0.40[a]	0.208±0.002
N116W/Q123-	1.0[a]	508.9±0.2[b]	527.9±0.1[b]	0.33[a]	0.236±0.004
E128-BDPY	0.9[a]	508.6±0.1[c]	533.7±0.1[c]	0.37[a]	0.215±0.001
N116W/E128-	0.9[a]	509.5±0.1[c]	533.0±0.1[c]	0.16[a]	0.238±0.001
N132-BDPY	1.0[a]	507.7±0.2[c]	531.9±0.1[b]	0.38[a]	0.217±0.001
N116W/N132-	0.8[a]	510.0±0.2[c]	532.0±0.7[b]	0.10[a]	0.255±0.001
K135-BDPY	0.9[a]	509.0±0.1[c]	531.3±0.1[b]	0.48[a]	0.235±0.001
N116W/K135-	0.9[a]	508.9±0.2[c]	531.3±0.3[b]	0.47[a]	0.237±0.001
Q123-LY	0.9[a]	427.1±1.2[c]	533.6±0.9[c]	0.164±0.013[b]	0.094±0.001
N116W/Q123-	0.9[a]	429.8±1.1[c]	533.5±0.5[c]	0.041±0.008[b]	0.049±0.001
E128-LY	0.9[a]	426.7±0.6[c]	533.5±0.8[c]	0.176±0.010[b]	0.106±0.001
N116W/E128-LY	0.9[a]	433.5±0.8[c]	531.5±1.8[c]	0.011±0.001[b]	0.049±0.001
N132-LY	0.9[a]	428.2±0.4[c]	532.1±1.0[c]	0.185±0.003[b]	0.141±0.001
N116W/N132-	0.9[a]	434.8±0.6[c]	534.6±0.5[c]	0.013±0.004[b]	0.097±0.001
K135-LY	1.0[a]	427.5±0.7[c]	532.8±0.3[c]	0.187±0.016[b]	0.138±0.007
N116W/K135-	0.8[a]	429.0±1.1[c]	532.6±0.1[c]	0.040±0.003[b]	0.147±0.002
Q123-Atto	0.7[a]	663.9±0.1[c]	679.1±1.2[c]	0.192±0.031[b]	0.223±0.001
N116W/Q123-	0.7[a]	665.4±0.3[c]	679.6±1.0[c]	0.099±0.014[b]	0.249±0.001
E128-Atto	0.5[a]	663.9±0.5[c]	678.9±0.6[c]	0.216±0.012[b]	0.221±0.001
N116W/E128-	0.7[a]	666.7±0.4[c]	677.8±0.4[b]	0.036±0.003	0.230±0.001
N132-Atto	0.5[a]	663.1±0.2[c]	678.3±0.1[c]	0.215±0.004[b]	0.212±0.001
N116W/N132-	0.8[a]	664.8±0.2[c]	678.1±0.5[c]	0.065±0.004[b]	0.221±0.002
K135-Atto	0.7[a]	660.1±0.1[c]	676.9±0.7[c]	0.222±0.003[b]	0.251±0.001
N116W/K135-	0.7[a]	664.4±0.2[c]	679.1±0.9[c]	0.075±0.005[b]	0.259±0.001
Q123-Cy5	0.8[a]	651.5[a]	666[a]	0.28[a]	0.257±0.001
N116W/Q123-	0.7[a]	651.5[a]	669[a]	0.25[a]	0.279±0.002
E128-Cy5	0.9[a]	650.5[a]	668.5[a]	0.28[a]	0.249±0.001
N116W/E128-	1.0[a]	651[a]	668.5[a]	0.25[a]	0.271±0.001
N132-Cy5	0.9[a]	650.5[a]	669.5[a]	0.32[a]	0.263±0.001
N116W/N132-	0.8[a]	650.5[a]	668.5[a]	0.29[a]	0.245±0.001
K135-Cy5	0.9[a]	651.5[a]	670[a]	0.28[a]	0.279±0.006
N116W/K135-	1.1[a]	651[a]	669[a]	0.21[a]	0.267±0.009

[*] Data are from Mansoor *et al, 2002 (5).* B_1 refers to the cysteine reactive label monobromobimane (mBBr), attached at the indicated residue using the same nomenclature as in the text. Absorbance values for this data set are reported as the average of two values, with an error of at most ± 1 nm. Emission λ_{max} and quantum yield values for this data set are reported as ± standard error of the mean for at least three measurements. ND = Not determined

[a] Measured once; [b] Measured twice; [c] Measured three or more times; [d] All anisotropy measurements were taken twice, ND = Not determined. All data reported as ± standard deviation

Supplemental Table 2: **Thermodynamic Characterization of a Subset of Labeled T4 Lysozyme Samples.**

Labeled Mutant	**T_m (°C)[b]**	**ΔT_m (°C)**	**$\Delta\Delta G$ (kcal/mol)[a]**
Wild-Type	52.14±0.04	---	---
E128-qBBr	46.67±0.21	- 2. 4	- 0.7
N116W/E128-qBBr	49.51±0.22	- 2. 6	- 0.8
E128-BDPY	49.22±0.09	- 2. 9	- 0.8
N116W/E128-BDPY	49.17±0.16	- 2. 9	- 0.8
E128-LY	49.83±0.18	- 2. 3	- 0.7
N116W/E128-LY	53.73±0.01	+ 1. 8	+ 0.5
E128-Atto	46.67±0.21	- 5. 4	- 1.6
N116W/E128-Atto	49.16±0.15	- 2. 9	- 0.8

[a] $\Delta\Delta G$ calculated using the approximation that $\Delta\Delta G = \Delta T_m * \Delta S_{WT}$ (*6*).

[b] Melting temperatures are an average of two measurements ± s.d.**,** except wild-type (3 measurements)

Supplemental Table 3: Susceptibility of Fluorophores to Quenching in the Presence of Various Amino Acids (Reported as Relative Quantum Yield[a]).

Fluorophore	Trp	Tyr-ME[b]	His	Phe	Met	Asp	Arg
qBBr	0.66	0.87	0.93	1.00	0.94	1.03	0.97
BODIPY 507	0.74	0.86	0.99	1.02	1.00	0.99	0.96
Lucifer Yellow	0.27	0.51	0.99	1.05	0.97	0.99	1.00
Atto-655	0.26	0.68	0.96	1.09	1.06	0.87	0.94
Cy5	0.93	1.02	0.98	1.01	0.99	0.97	1.01

[a] Relative quantum yields were calculated as the integrated fluorescence emission in the presence of 30 mM of the indicated amino acid, divided by integrated emission intensity from the label in buffer alone (See Methods). The amino acids used are indicated by their three-letter abbreviation. Results are the average of two independent assays.

[b] Because of problems with solubility, tyrosine methyl ester was used for the tyrosine quenching experiments.

Supplemental Table 4: **One and Two-Exponential Lifetime Analysis of the Fluorescence Decay Measurements of the Labeled Lysozyme Samples.**[a]

Mutant	τ_1 (ns)	α_1 (ns)	τ_2 (ns)	α_2	χ^2	$\langle\tau\rangle$ (ns)[b]
*Q123-B_1	9.9 ± 0.1	0.9 ± 0.1	1.0e-2 ± 9.8e-3	0.1 ± 0.1	1.3 ± 0.2	8.9 ± 0.4
*N116W/Q123-B_1	7.3 ± 0.1	0.8 ± 0.1	0.8 ± 0.3	0.3 ± 0.1	1.0 ± 0.2	5.6 ± 0.3
*E128-B_1	8.7 ± 0.0	1.0 ± 0.1	0.9 ± 0.1	0.1 ± 4.0e-2	0.9 ± 0.1	8.5 ± 0.1
*N116W/E128-B_1	4.9 ± 0.0	0.3 ± 0.0	0.9 ± 0.0	0.7 ± 0.0	0.9 ± 0.0	2.1 ± 6.9e-3
*N132-B_1	12.1 ± 0.3	0.9 ± 0.0	0.8 ± 0.5	0.1 ± 0.0	1.0 ± 0.1	10.9 ± 0.4
*N116W/N132-B_1	8.9 ± 0.6	0.7 ± 0.1	1.4 ± 0.7	0.4 ± 0.1	0.9 ± 0.1	6.2 ± 0.6
*K135-B_1	8.8 ± 0.1	0.7 ± 0.1	0.9 ± 0.3	0.3 ± 0.1	0.8 ± 0.1	6.5 ± 0.3
*N116W/K135-B_1	6.1 ± 0.1	0.8 ± 0.1	0.7 ± 0.0	0.3 ± 0.1	0.9 ± 0.1	4.8 ± 0.03
Q123-qBBr	4.87 ± 0.12	0.42 ± 0.03	1.93 ± 0.02	0.58 ± 0.03	0.99 ± 0.12	3.16 ± 0.04
N116W/Q123-qBBr	3.91 ± 0.04	0.43 ± 0.01	1.03 ± 0.06	0.57 ± 0.01	0.85 ± 0.06	2.27 ± 0.08
E128-qBBr	6.55 ± 0.26	0.55 ± 0.02	2.82 ± 0.12	0.45 ± 0.02	0.84 ± 0.05	4.87 ± 0.14
N116W/E128-qBBr	3.91 ± 0.19	0.39 ± 0.02	0.67 ± 0.03	0.61 ± 0.02	1.21 ± 0.07	1.94 ± 0.03
N132-qBBr	7.61 ± 0.18	0.48 ± 0.01	2.37 ± 0.18	0.52 ± 0.01	0.91 ± 0.12	4.90 ± 0.12
N116W/N132-qBBr	4.34 ± 0.04	0.56 ± 0.02	1.17 ± 0.09	0.44 ± 0.02	0.72 ± 0.06	2.95 ± 0.08
K135-qBBr	4.45 ± 0.11	0.37 ± 0.01	1.64 ± 0.05	0.63 ± 0.01	0.94 ± 0.19	2.67 ± 0.09
N116W/K135-qBBr	3.49 ± 0.06	0.41 ± 0.01	1.21 ± 0.01	0.59 ± 0.01	0.83 ± 0.01	2.14 ± 0.05
Q123-BDPY	3.39 ± 0.01	0.91 ± 0.01	8.85 ± 0.54	0.09 ± 0.01	1.01 ± 0.02	3.75 ± 0.14
N116W/Q123-BDPY	2.62 ± 0.20	0.81 ± 0.08	7.11 ± 1.19	0.19 ± 0.08	1.02 ± 0.05	3.13 ± 0.11
E128-BDPY	3.28 ± 0.03	1.00	---	---	1.16 ± 0.02	3.41 ± 0.01
N116W/E128-BDPY	1.73 ± 0.03	0.73 ± 0.02	4.32 ± 0.14	0.27 ± 0.02	1.03 ± 0.10	2.42 ± 0.02
N132-BDPY	2.97 ± 0.02	0.95 ± 0.01	11.01 ± 0.78	0.05 ± 0.01	0.99 ± 0.06	3.35 ± 0.03
N116W/N132-BDPY	1.17 ± 0.07	0.52 ± 0.02	3.15 ± 0.06	0.48 ± 0.02	0.84 ± 0.01	2.11 ± 0.06
K135-BDPY	1.62 ± 0.81	0.33 ± 0.11	4.86 ± 0.66	0.67 ± 0.11	1.03 ± 0.09	3.87 ± 0.26
N116W/K135-BDPY	2.24 ± 0.59	0.42 ± 0.16	5.19 ± 0.57	0.57 ± 0.16	1.01 ± 0.03	3.94 ± 0.12
Q123-LY	7.32 ± 0.03	1.00	---	---	0.99 ± 0.03	7.32 ± 0.04
N116W/Q123-LY	1.78 ± 0.17	0.26 ± 0.01	5.77 ± 0.05	0.74 ± 0.01	1.04 ± 0.05	4.74 ± 0.06
E128-LY	7.27 ± 0.09	1.00	---	---	1.00 ± 0.05	7.27 ± 0.09
N116W/E128-LY	1.36 ± 0.13	0.15 ± 0.01	6.75 ± 0.06	0.85 ± 0.01	0.99 ± 0.07	5.98 ± 0.04
N132-LY	7.63 ± 0.03	1.00	---	---	0.97 ± 0.02	7.63 ± 0.03
N116W/N132-LY	1.49 ± 0.19	0.19 ± 0.01	6.96 ± 0.06	0.81 ± 0.01	1.01 ± 0.09	5.92 ± 0.04
K135-LY	7.49 ±0 .05	1.00	---	---	1.04 ± 0.02	7.49 ± 0.05
N116W/K135-LY	1.59 ± 0.29	0.16 ± 0.02	6.48 ± 0.06	0.84 ± 0.03	0.98 ± 0.05	5.70 ± 0.11
Q123-Atto	2.23 ± 0.01	1.00	---	---	1.13 ± 0.02	2.23 ± 0.01
N116W/Q123-Atto	2.12 ± 0.01	1.00	---	---	1.10 ± 0.09	2.12 ± 0.01
E128-Atto	2.14 ± 0.01	1.00	---	---	1.08 ± 0.06	2.14 ± 0.01
N116W/E128-Atto	2.01 ± 0.01	1.00	---	---	1.08 ± 0.04	2.01 ± 0.01
N132-Atto	2.07 ± 0.03	1.00	---	---	1.09 ± 0.09	2.07 ± 0.03
N116W/N132-Atto	1.82 ± 0.02	1.00	---	---	1.07 ± 0.03	1.82 ± 0.02
K135-Atto	2.41 ± 0.02	1.00	---	---	1.05 ± 0.07	2.41 ± 0.02
N116W/K135-Atto	1.97 ± 0.01	1.00	---	---	1.13 ± 0.03	1.97 ± 0.01

* Data are from Mansoor *et al, 2002 (5)*. B_1 refers to the cysteine reactive label monobromobimane (mBBr), attached at the indicated residue using the same nomenclature as in the text. The $\langle\tau\rangle$ values reported for this subset of data represent the average of two sets of lifetimes ± the standard error of the mean.

[a] Excitation and emission collected as reported in Methods. The average of three sets of lifetime data are reported ± the SEM.

Abbreviations: τ_1, τ_2, fluorescence lifetimes in nanoseconds; α_1, α_2, normalized pre-exponential factors such that $\alpha_1 + \alpha_2 = 1.0$; χ^2, chi-squared value of the fit.

[b] $\langle\tau\rangle = \alpha_1\tau_1 + \alpha_2\tau_2$, the amplitude-weighted average fluorescence lifetime. The $\langle\tau\rangle$ values reported in this table represent the average of the three sets of lifetimes ± the standard error of the mean (except for qBBr which was an average of two).

Supplemental Table 5: **Fraction of fluorophores which have formed a static complex, the fraction of fluorophores which are dynamically quenched and the fraction of fluorophores which fluoresce unquenched.**

$\gamma = \gamma_{DQ} + \gamma_F$

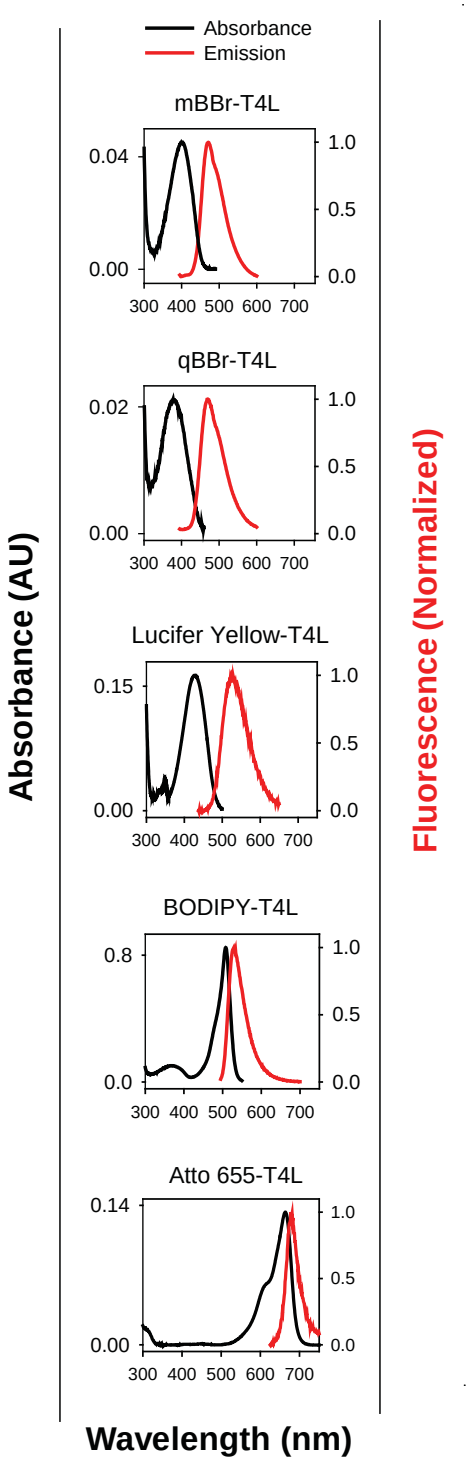
Sample Pair	Fraction Complexed (1–γ)	Fraction Open (γ) [a]	Dynamically Quenched γ (γ_{DQ}) [b]	Unquenched γ (γ_F) [c]
*123-B_1	0.03 ± 0.13	0.97 ± 0.13	0.36 ± 0.16	0.61 ± 0.09
*128-B_1	-0.08 ± 0.02	1.08 ± 0.02	0.81 ± 0.02	0.27 ± 0.01
*132-B_1	0.66 ± 0.04	0.34 ± 0.04	0.15 ± 0.05	0.19 ± 0.03
*135-B_1	-0.14 ± 0.06	1.14 ± 0.06	0.30 ± 0.08	0.84 ± 0.06
123-qBBr	0.18 ± 0.05	0.82 ± 0.05	0.23 ± 0.06	0.59 ± 0.04
128-qBBr	0.51 ± 0.02	0.49 ± 0.02	0.30 ± 0.03	0.20 ± 0.01
132-qBBr	0.76 ± 0.05	0.24 ± 0.05	0.10 ± 0.06	0.14 ± 0.03
135-qBBr	0.03 ± 0.06	0.97 ± 0.06	0.19 ± 0.08	0.78 ± 0.06
123-BDPY	0.02 ± 0.06	0.98 ± 0.06	0.16 ± 0.09	0.81 ± 0.06
128-BDPY	0.40 ± 0.01	0.60 ± 0.01	0.18 ± 0.01	0.43 ± 0.01
132-BDPY	0.64 ± 0.01	0.36 ± 0.01	0.13 ± 0.02	0.23 ± 0.01
135-BDPY	0.05 ± 0.07	0.95 ± 0.07	-0.02 ± 0.12	0.97 ± 0.10
123-LY	0.58 ± 0.01	0.42 ± 0.01	0.15 ± 0.01	0.27 ± 0.01
128-LY	0.92 ± 0.01	0.08 ± 0.01	0.01 ± 0.01	0.07 ± 0.01
132-LY	0.93 ± 0.01	0.07 ± 0.01	0.01 ± 0.01	0.05 ± 0.01
135-LY	0.72 ± 0.01	0.28 ± 0.01	0.07 ± 0.01	0.21 ± 0.01
123-Atto	0.46 ± 0.01	0.54 ± 0.01	0.03 ± 0.01	0.51 ± 0.01
128-Atto	0.82 ± 0.01	0.18 ± 0.01	0.01 ± 0.01	0.17 ± 0.01
132-Atto	0.64 ± 0.01	0.36 ± 0.01	0.04 ± 0.01	0.31 ± 0.01
135-Atto	0.66 ± 0.01	0.34 ± 0.01	0.06 ± 0.01	0.28 ± 0.01
*72/76W-B_1	0.58 ± 0.04	0.42 ± 0.04	0.24 ± 0.05	0.18 ± 0.02
*124/126W-B_1	0.15 ± 0.04	0.85 ± 0.04	0.66 ± 0.04	0.19 ± 0.01
*133/138W-B_1	0.52 ± 0.04	0.48 ± 0.04	0.26 ± 0.04	0.22 ± 0.02

[a] $\gamma = \left(\frac{F_w}{F_0}\right)\left(\frac{\tau_0}{\tau_w}\right)$, where F_w and τ_w represent the relative quantum yield and fluorescence lifetime of the fluorophore in the presence of the Trp quencher, and F_0 and τ_0 represent the relative quantum yield and fluorescence lifetime in the absence of the Trp quencher.

[b] The component of γ that is dynamically quenched is calculated as: $\gamma_{DQ} = \left(1 - \frac{\tau_w}{\tau_0}\right) \cdot \gamma$

[c] The component of γ that fluoresces unquenched is calculated as: $\gamma_F = \frac{\tau_w}{\tau_0} \cdot \gamma$

[*] The primary data to perform this analysis for the mBBr-labeled samples are taken from Mansoor *et al*., 2002 (*5*).



Supplemental Figure 1. Absorbance and fluorescence emission spectra of each fluorophore used in this study. Absorbance spectra (black, left axis) and normalized fluorescence spectra (red, right axis) of each fluorescent label while attached to T4 lysozyme.