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COMMENTARY

An Ionization/Recombination Mechanism for Complexity of the Fluorescence of Tryptophan in Proteins

The decay of the fluorescence of tryptophan in proteins is very rarely described by a single exponential term even when the protein has only a single tryptophan residue at an interior position.[1] Two components may suffice in some cases, but often three or even more components are indicated. Typically the decay is described by components of 0.5−1.5 and 3−5 ns decay times with similar amplitudes.

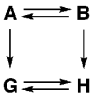
Interpretations of this "heterogeneity" fall into two classes. One is in terms of ground-state heterogeneity, i.e., multiple conformational states of the protein[1,2] sometimes termed the "rotamer" model.[3,4] In this model the decay components refer to protein conformational states each with their own fluorescence lifetime. The interconversion of these species must be slow in comparison with the fluorescence lifetime of about 10 ns, and the two species must have similar free energies in order to result in comparable decay amplitudes. The ratio of the amplitudes is the conformational equilibrium constant.

Another class of interpretation of this heterogeneity is in terms of an excited-state process. In this model the excited indole chromophore converts to a new fluorescent species after electronic excitation. One such process is relaxation of mobile polar groups due to the increased dipole moment in the excited electronic state.[5−8] If this relaxation is very rapid or very slow, then only a single-exponential decay will be observed. However, if the relaxation process is on the same time scale as the excited-state lifetime and if the relaxation product is fluorescent but has properties distinct from the unrelaxed species, a biexponential decay is possible.

There is considerable evidence from model studies in favor of each of these hypotheses. On the other hand, there are difficulties with each of them either internally or as applied to protein fluorescence. Neither has been unequivocally proven to apply for any specific protein. We have proposed[9] a third hypothesis that, like the "relaxational" model, is homogeneous in nature. This involves ionization of the excited tryptophan residue due to collisional transfer of an electron to a neighboring amino acid side chain. For buried tryptophan residues the postulated electron acceptor is the side chain of a neighboring residue. Two features distinguish the photophysics of tryptophan in proteins from the behavior in solution. The first is the constraint against diffusional separation of the radical anion and radical cation. The second is the lack of hopping of the electron from one equivalent site to another. The constrained proximity of the radical cation and radical anion permits subsequent recombination luminescence. For the case of a single electron acceptor this results in a double-exponential fluorescence decay.

The subject of this Commentary is the presentation to a wider audience of the arguments supporting this novel interpretation of fluorescence decay behavior. We show that this model fits the available experimental data. It makes specific, testable predictions concerning the effect of mutational changes at neighboring residues and with respect to addition of external quenching agents. It suggests that protein photophysics, combined with site-directed mutagenesis and structural studies, can be a good way of studying fast electron-transfer reactions in proteins in considerable detail.

The general kinetic scheme



includes all of the processes discussed above as special cases. In this scheme **A** is the excited tryptophan residue which decays to the ground state **G** by both radiative and nonradiative processes ($k_{\mathbf{A}} = k_{\mathbf{A}}^{o} + k_{\mathbf{A}}^{nr}$). The processes **A** → **B**, **B** → **A**, and **B** → **H** have different interpretations in the different models. The ground-state interconversion process **G** ⇄ **H** is described by an equilibrium $K_{\mathbf{GH}} = [\mathbf{H}]/[\mathbf{G}] = k_{\mathbf{GH}}/k_{\mathbf{HG}}$.

In the heterogeneous population or rotamer model the interconversion rates k_{AB} and k_{BA} are slow compared to the decay rates $k_{\mathbf{A}}$ and $k_{\mathbf{B}}$ ($k_{\mathbf{AB}} \ll k_{\mathbf{A}}, k_{\mathbf{B}}$). The relative populations of **G** and **H**, and thus the amplitudes of the decay components, are described by the equilibrium constant $K_{\mathbf{GH}}$. Both decay processes $k_{\mathbf{A}}$ and $k_{\mathbf{B}}$ must have radiative components and the value of $k_{\mathbf{A}}$ must differ from that of $k_{\mathbf{B}}$ in order for a double-exponential decay to be observed. The two species **G** and **H** may have different absorption spectra, and **A** and **B** may have different emission spectra. In this model the observed decay is $\alpha(\lambda)\, A(t) + \beta(\lambda)\, B(t)$ where the weighting factors depend on the emission wavelength if the two emission spectra differ and on the excitation wavelength if the two absorption spectra differ.

In the relaxational model the equilibrium $K_{\mathbf{GH}} \ll 1$, $k_{\mathbf{AB}}$ is on the order of $k_{\mathbf{A}}$ and $k_{\mathbf{BA}} \ll k_{\mathbf{AB}}$. Both $k_{\mathbf{A}}$ and $k_{\mathbf{B}}$ must have radiative components and either $k_{\mathbf{A}} \neq k_{\mathbf{B}}$ or the two species must have different emission spectra (or both). Again, both $\mathbf{A}(t)$ and $\mathbf{B}(t)$ contribute in a way that may depend on the emission wavelength or on the emission wavelength or on the excitation wavelength or on both.

If the species **B** is "dark", i.e., $k_{\mathbf{B}}$ has no radiative component, then the **A** → **B** → **H** reaction is just another radiationless decay path which increases the rate of decay of species **A**. However, if there is a reverse reaction, **B** → **A**, then the presence of a "dark state", **B**, results in a double-exponential decay for the population of **A**. The solution of this kinetic scheme for the case $K_{\mathbf{GH}} = 0$ ($A(0) = 1$ and $B(0) = 0$) is[10,11] $A(t) = \alpha \exp(-k_1 t) + (1 - \alpha) \exp(-k_2 t)$. Using the definitions[11] $X = k_{\mathbf{A}} + k_{\mathbf{AB}}$, $Y = k_{\mathbf{B}} + k_{\mathbf{BA}}$ and $Z^2 = k_{\mathbf{BA}} k_{\mathbf{AB}}$, we have $k_{1,2} = [(X + Y) \pm [(X - Y)^2 + 4Z^2]^{1/2}]/2$ ($k_1 > k_2$) and $\alpha = (X - k_2)/(k_1 - k_2)$. This analytic solution will be illustrated and applied below. The decay does not depend on either emission or excitation wavelength. Note that the amplitudes of the fluorescence decay, α and $1 - \alpha$, depend on the rate

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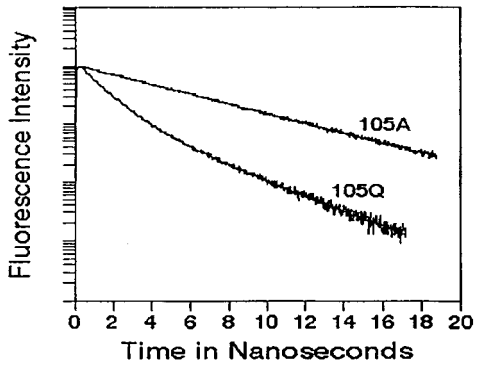
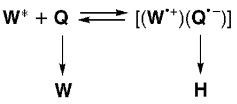


FIGURE 1. Logarithmic fluorescence decay of bacteriophage T4 lysozyme YWY mutants with glutamine at position 105 (105Q) and YWY/Q105A with alanine at position 105 (105A). Data taken from ref 17.

constants and not on any ground-state equilibrium. In order for the reverse process to have an appreciable rate compared to the forward process, **B** and **A** must have similar energies. To be dark and of high energy, **B** must be a distinct chemical species. (The environment of the chromophore can only influence the radiationless decay or have a minor effect on the radiative decay.) To figure in this kinetic scheme this species must be formed rapidly from **A**. We propose the contact radical ion pair formed by collisional ionization as a candidate for this proposed dark intermediate species. In this case the abstract kinetic scheme above becomes



where **W** and **W*** are the ground and excited states of tryptophan, **Q** is a collisional electron acceptor, and **H** is either the ground state of tryptophan or another low-energy species (e.g., the triplet state). $\mathbf{W}^{\bullet+}$ is the radical cation of tryptophan and $\mathbf{Q}^{\bullet-}$ is the radical anion of the species accepting the electron. The consecutive forward process $\mathbf{W}^* + \mathbf{Q} \rightarrow [(\mathbf{W}^{\bullet+})(\mathbf{Q}^{\bullet-})] \rightarrow \mathbf{H}$ leads to quenching of the excitation and is the usual mechanism of fluorescence quenching. The species **Q** will be referred to as the quencher. For buried tryptophan residues, **Q** will be the side chain of some other amino acid. The first case to be discussed involves the amino acid glutamine as the quenching species for which the standard symbol is, coincidentally, Q.

To illustrate the applicability of this kinetic scheme in the case of tryptophan fluorescence, we take the specific case of bacteriophage T4 lysozyme in which the only tryptophan residue present is at the buried location 138. The tryptophan residues in the wild-type sequence at positions 126 and 158 have been replaced by tyrosine.[9,12] This protein is designated "105Q" because of the presence of a glutamine residue at this position. Its fluorescence decay is the curve in Figure 1. A least-squares fit of two exponential components to these data yields $\alpha = 0.79$, $k_1 = 1/1.1$ ns $= 0.91$ ns^{-1}, $1 - \alpha = 0.21$, and $k_2 = 1/3.1$ ns $= 0.32$ ns^{-1}. As discussed below[13] the fundamental rate constant $k_{\mathbf{A}}$, the decay rate of **W*** in the absence of quenching, is 0.2 ns^{-1}. The decay data then yield the forward electron-transfer rate, $k_{\mathbf{AB}} = 0.6$ ns^{-1}, the reverse electron-transfer rate $k_{\mathbf{BA}} = 0.1$ ns^{-1}, and the rate of decay of the contact radical ion pair $k_{\mathbf{B}} = 0.35$ ns^{-1}.

At this point this model is at least as good as the other previously proposed models in terms of fitting the data. More complex fluorescence decay behavior can be accommodated by assumption of more than one electron acceptor site. Many amino acids are known to cause collisional quenching of the fluorescence of tryptophan in solution probably by an electron-transfer mechanism.[9,14] One of the moderate quenchers of tryptophan fluorescence is glutamine. Glutamine-105 of bacteriophage T4 lysozyme is hydrogen bonded to the indole NH residue of tryptophan. Changing glutamine-105 to alanine results in a 3-fold increase in fluorescence intensity[9] with no change in the structure.[15] This is consistent with the lower quenching efficiency of alanine relative to glutamine. The time dependence of the fluorescence of this mutant protein ("105A") is compared to that of the protein with a glutamine residue at position 105 in Figure 1. This decay is essentially single-exponential with a lifetime of 5.1 ns.[9]

This result strongly suggests that there is a relationship between the quenching of the fluorescence of tryptophan residues in proteins and the observation of multiple components in the fluorescence decay. This is consistent with the observation that tryptophan residues that are highly quenched often exhibit a complex time dependence in their fluorescence decay behavior.[16] The elucidation of this connection led to the ionization/recombination hypothesis outlined above. In retrospect, given the nature of fluorescence quenching and the photophysical behavior of tryptophan, this explanation should have been obvious.

Collisional quenching of fluorescence is often interpreted in terms of an electron-transfer process.[17] The bimolecular collisional rate constants for quenching of tryptamine by amino acids has been determined.[9,14] These values range from 2.7×10^9 M^{-1} s^{-1} for histidinium to 1×10^8 M^{-1} s^{-1} for alanine with glutamine being intermediate (5×10^8 M^{-1} s^{-1}). There is a strong correlation (>0.95) between the bimolecular collisional rate constants and the ability of these same species to scavenge electrons in a pulse radiolysis experiment, i.e., the rate at which electrons attach to form the radical anion from an aqueous solution. This strongly suggests that the mechanism of quenching of fluorescence of tryptophan by amino acid side chains is collisional electron transfer. The quenching of tryptophan by acrylamide has been interpreted in this fashion.[18]

Tryptophan and its analogues are known to photoionize in aqueous solution.[5,19] Excitation at 280 nm results in about an 8% quantum yield for formation of electrons. This yield increases as the wavelength of excitation is decreased, being ca. 25% at 220 nm. These measurements refer to the net yield of electrons; the instantaneous value may be higher.

The phenomenon of recombination luminescence is well established for aromatic organic species.[20,21] This process is usually described for fluorescent organic compounds in solutions at low temperature ("glasses"). After illumination of the frozen sample, recombination luminescence is associated with either thawing the glass or illumination with infrared radiation in the region of absorption of solvated electrons.

The fact that tryptophan ionizes in polar solvent solutions might lead to resolvable recombination luminescence depending on the nature of electron mobility in the solution, the time scale of recombination, and the energetics and time scale of solvent relaxation. It is in fact observed that the decay of the fluorescence of 3-methylindole in water and acetonitile differs in form from that observed in cyclohexane. Specifically, analysis of the decay data in terms of a Gaussian

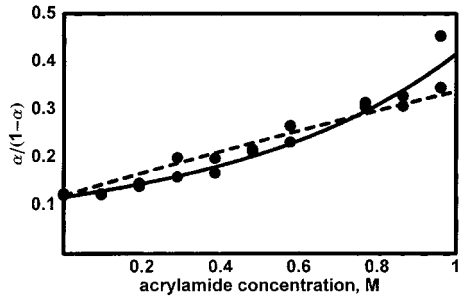


FIGURE 2. Variation in the ratio $\alpha/(1-\alpha)$ for bacteriophage T4 "YWY"/Q105A with added acrylamide. There are two data points for each concentration, some of which overlap.

distribution results in a finite width in the case of the polar solvents but not in the cyclohexane case.[22] This is consistent with ionization.

These results show that the individual steps for the proposed ionization/recombination luminescence mechanism have been demonstrated. On the other hand, the steps prerequisite to the multiple conformational state (rotamer) hypothesis and the relaxational hypothesis have also been amply demonstrated in terms of experiments performed on tryptophan and simple peptides in solution. The fact that the kinetic model based on ionization/recombination fits the observed fluorescence data proves only that this model is not inconsistent with the observations.

According to the multiple conformational state explanation of the heterogeneity of tryptophan fluorescence, the ratio of the decay amplitudes, $\alpha/(1-\alpha)$, is equal to the equilibrium constant $K_{\mathbf{GH}}$. In contrast, for the model proposed here, the amplitude ratio $\alpha/(1-\alpha)$ depends on the decay and interconversion rate constants in a complex but monotonic fashion (specifically, $\alpha/(1-\alpha) = (X - k_2)/(k_1 - X)$ where $X = k_{\mathbf{A}} + k_{\mathbf{AB}}$ and k_1 and k_2 depend in a complex way on all the rate constants).

If an extrinsic quencher, e.g., acrylamide, is added to a solution of a protein containing tryptophan, the values of $k_{\mathbf{A}}$ and $k_{\mathbf{B}}$ are increased (perhaps by different amounts). In the multiple conformational state model, each conformer will have its lifetime shortened (perhaps in distinct ways),[2] but unless there is binding of the quencher to the protein and that binding is highly preferential to one conformation over another, the equilibrium ratio $\alpha/(1-\alpha)$ will remain constant. In the ionization/recombination model the ratio $\alpha/(1-\alpha)$ will, in general, vary with addition of quencher. The variation of $\alpha/(1-\alpha)$ as a function of acrylamide concentration for the 105Q variant of T4 lysozyme is shown in Figure 2.[23] This quantity is clearly not constant. This is inconsistent with the multiple conformation state hypothesis unless binding of acrylamide occurs with a strong preference to one of the conformational states. This has been looked for in the present case by observing that there was no effect of addition of the analogue propionamide on the fluorescence decay.

The relevant quantity that determines the variation of $\alpha/(1-\alpha)$ with concentration of quencher is the differential quenching of the excited singlet state of tryptophan, **W*** or **A**, and the contact radical ion pair, $[(\mathbf{W}^{\bullet+})(\mathbf{Q}^{\bullet-})]$ or **B**. This quenching can be described in the usual pseudo-first-order (Stern−Volmer) fashion: $k_{\mathbf{A}} = k_{\mathbf{A}0} + k_{\mathbf{qA}}[\mathbf{Q}]$ where $[\mathbf{Q}]$ is the concentration of collisional quencher with bimolecular quenching constant $k_{\mathbf{qA}}$. The expression for $\alpha/(1-\alpha)$ involves X_0, Y_0, and Z^2 (all of which can be determined from the $[\mathbf{Q}] = 0$ data) and one new parameter, $\Delta_q = k_{\mathbf{qA}} - k_{\mathbf{qB}}$. A reasonable value ($\Delta_q = 0.65\ M^{-1}\ s^{-1}$) results in the solid curve in Figure 2. This shows that the ionization/recombination hypothesis together with the Stern−Volmer model is quantitatively consistent with the data. The fit of the multiple conformational state picture with a differential binding model to the data gives the dashed line in Figure 2. This has curvature opposite that of the ionization/recombination model. More importantly this fit requires that the binding be very preferential, 20-fold larger for one species over the other. This is unreasonable.

The ionization recombination model has been generalized to the case of two quenchers[24] resulting in a triple exponential and has been applied to several cases with reasonable results.[25] Given the ubiquitous nature of side chain quenchers,[9,14] more complex cases of this type are probably common. Given the propensity of tryptophan to ionize and the commonly accepted mechanism of collisional quenching of aromatic excited states, plus the observed variation of the ratio $\alpha/(1-\alpha)$, the ionization/recombination mechanism seems the most likely of the alternative descriptions.

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(13) A double-exponential fluorescence decay such as the one analyzed above contains only three pieces of information, the two decay rates and the relative amplitude of the two decay components. To extract four rate constants from these data, it is necessary to have additional information. This is provided by the T4 lysozyme 105-A variant where, by hypothesis, the quenching residue has been removed. The observed fluorescence lifetime in the absence of Q (glutamine) at position 105 provides the value of k_A directly. The analytic solution for $A(t)$ in the presence of the quencher can be inverted to provide X, Y, and Z directly from the data: $X = \alpha(k_1 - k_2) + k_2$, $Y = (k_1 + k_2) - X$, $Z = [(k_1 - k_2)^2 - (X - Y)^2]/4$. Since $X = k_A + k_{AB}$, we obtain k_{AB} from X and k_A. From $Z^2 = k_{AB}k_{BA}$ and k_{AB} we obtain k_{BA}. From Y and k_{BA} we obtain k_B.
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