A Reversible "Dark State" Mechanism for Complexity of the Fluorescence of Tryptophan in Proteins

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A model is described for the complex fluorescence of tryptophan in proteins involving a reversible process between the excited singlet state of tryptophan and a "dark" (nonemissive) state of high energy. A specific hypothesis for the nature of this dark state is given that involves rapid ionization of the excited tryptophan followed by indole nitrogen proton transfer on the nanosecond time scale. The general kinetic scheme involved predicts that there will be a change in amplitude of the decay components when an external quencher is added. This experiment is presented and shown to be consistent with the model. It is argued that this result is inconsistent with any static conformational heterogeneity hypothesis.

The decay of the fluorescence of a chromophore in fluid solution is usually described by an exponential decay. The decay of the fluorescence of tryptophan in proteins is very rarely described as an exponential decay. This is the case even when the protein in question has only a single tryptophan residue located at an interior position. It is possible to describe the decay of the fluorescence of tryptophan in proteins as the sum of several exponential terms.[1] Two components may suffice in some cases but often three or even more components are indicated. Typically, the decay has 60% with a lifetime of 0.5−1.5 ns and 40% with a 3−5 ns decay time in the simplest (two-component) cases.

The interpretations of this "heterogeneity" of protein fluorescence can be divided into two categories. One interpretation is in terms of ground-state heterogeneity, i.e., multiple conformational states of the protein.[1,2] This is sometimes termed the "rotamer" model because of its application to the multiple component fluorescence decay behavior of tryptophan in solution where several side chain conformations are possible.[3−12] In this model the discrete decay components refer to the discrete 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. The amplitudes of the normalized fluorescence decay represent the relative fraction of each conformer in this interpretation. For the two-component example given above, there must be roughly equal amounts of each conformation present. Assuming that these are at equilibrium, the two species must have similar free energies.

Another type of interpretation of this "heterogeneity" is in terms of an excited-state process rather than ground-state heterogeneity. The general idea is that the excited indole chromophore can convert from its initial form to a new fluorescent species after electronic excitation. One concrete description of such a process is reorientational relaxation of the mobile polar groups surrounding the indole chromophore associated with the much higher dipole moment of the molecule in the excited electronic state than in the ground electronic state.[13−16] 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 of the initially excited species, then a biexponential decay is possible if the relaxation product is fluorescent but has properties distinct from the unrelaxed species.

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. For example, the relaxational model predicts a rise in the fluorescence when observed on the red-edge of the emission spectrum. This is clearly seen for indolyl chromophores (e.g., 3-methylindole) in viscous polar solvents but has never been observed for any single tryptophan protein. The "rotamer" model, when applied to proteins with buried tryptophan residues, is subject to several difficulties. One is that multiple orientational occupancy is not indicated by the X-ray or NMR data. Another is that it is difficult, at least in some cases (including trp-138 of T4 lysozyme) to build a molecular model for the protein with alternative orientations of the chromophore that are expected to be persistent as discrete species with the requesite multiple nanosecond residence time. Direct demonstration of the heterogeneity of the ground-state population would be obtained if the fluorescence decay amplitudes (but not lifetimes) changed with excitation wavelength. This has not been demonstrated. These are not insurmountable difficulties, however. But this model has not been proved.

We have proposed a third hypothesis that is a variant of the second or "relaxational" model.[17] This involves reversible formation of a "dark" (nonfluorescent) species that returns to the emissive state. Specifically, we proposed electron transfer from the excited tryptophan residue to a neighboring quenching side chain residue. The resulting contact radical ion pair cannot separate by diffusion because of the covalent linkage of both partners to the protein backbone. This distinguishes the photophysics of tryptophan in proteins from the behavior of indolyl

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chromophores in solution. The constrained proximity of the radical cation and radical anion permit subsequent recombination luminescence. For the case of a single electron acceptor this results in a double-exponential fluorescence decay. For multiple electron acceptors this results in more complex decay behavior.

This ionization/recombination model by itself is subject to the general criticism that photoionization, even that requiring collision between an excited tryptophan residue and a neighboring side chain, is expected to be very rapid. In the absence for the need for collision this process occurs on the femtosecond time scale. For indole in aqueous solution there is evidence that ionization is competitive with internal conversion. Collision times for residues neighboring the indole ring are expected to be on the picosecond time scale. These processes, instantaneous or collisional ionization, are expected to be too fast to be relevant to the time dependence of the fluorescence process. Since the pK_a of the radical cation of tryptophan is known to be 4.2,[18] an obvious "slow" step is the loss of the indole N−H proton to a neighboring base (or the aqueous solvent). This hypothesis is discussed.

The key features of this model that distinguish it from previous hypotheses are (1) the formation of a dark intermediate and (2) the reversibility of the process on the nanosecond time scale. These are the only kinetically relevant aspects of the argument. The nature of the dark intermediate (and the corresponding forward and reverse steps) can have several forms although certain physical constraints are implied by this model.

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 considerable detail.

The basic features of the model are first presented followed by evidence that supports the plausibility of this hypothesis. An experiment based on quenching by added acrylamide is then described. The results of this experiment demonstrate that the basic model is correct in terms of the kinetic scheme involved. The conformational heterogeneity hypothesis is not consistent with the results of this experiment unless very preferential binding is invoked.

Experimental Section

The fluorescence data presented here was all obtained by single photon, delayed coincidence counting methods described previously.[17,19] The preparation of the T4 lysozyme variants has also been discussed previously.[17,19] Data for two variants of bacteriophage T4 lysozyme are discussed here. Both contain only a single tryptophan residue at interior position 138. Residues 126 and 158, which are trp residues in the wild-type protein, have been replaced by tyrosine residues in each case. The two species studied differ in the presence of a glutamine residue at position 105 (as in the wild-type) or an alanine residue at that position. They are designated 105Q and 105A, respectively.

The fluorescence data for these proteins is analyzed by the maximum entropy method to determine the number of decay components. Some examples of the results have been given. In all of the cases we have analyzed, including the cases under consideration, two or three well-resolved peaks are observed in the resulting distribution of lifetimes. The data are subsequently analyzed with a sum of exponential terms, the number of such terms corresponding to the number of peaks in the maximum entropy distribution.

Description of the Proposed Mechanism: Reversible Excited-State Reactions with Dark Intermediates

General kinetic Scheme 1 includes all of the hypothetic

SCHEME 1

$$\begin{array}{ccc} \mathbf{A} & \rightleftarrows & \mathbf{B} \\ \downarrow & & \downarrow \\ \mathbf{G} & \rightleftarrows & \mathbf{X} \end{array}$$

processes discussed above as special cases. In this scheme A is the excited tryptophan residue. This may decay to the ground-state G by both radiative and nonradiative processes. The processes A → B and B → A (with rate constants k_{AB} and k_{BA}) have different interpretations in the different models. The process B → X also has different interpretations in each model and is assigned the rate constant k_B in this general scheme. The ground-state interconversion process GVX is described by an equilibrium $K_{GX} = [X]/[G] = k_{GX}/k_{XG}$.

In the heterogeneous population or "rotamer" model the interconversion rates k_{AB} and k_{BA} are small compared to the decay rates k_A and k_B. The relative populations of G and X, and thus the amplitudes of the decay components, are described by the equilibrium constant K_{GX}. Both decay processes k_A and k_B must have radiative components and the value of k_A must differ from that of k_B in order for a double-exponential decay to be observed. The two species G and X 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 $K_{GX} \ll 1$ and $k_{AB} \gg k_{BA}$. To observe a double-exponential, both k_A and k_B must have radiative components and either $k_A \neq k_B$ or the two species must have different emission spectra (or both). Again, both $A(t)$ and $B(t)$ contribute in a way that may depend on the emission wavelength or on the excitation wavelength or both.

If the species B is "dark", i.e., k_B has no radiative component, then the A → B → X reaction is just another first-order radiationless decay path that 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 $K_{GX} = 0$ ($A(0) = 1$ and $B(0) = 0$), is[20,21]

$$A(t) = \alpha \exp(-k_1 t) + (1 - \alpha) \exp(-k_2 t) \qquad (1a)$$

$$B(t) = [k_{AB}/(k_1 - k_2)][\exp(-k_2 t) - \exp(-k_1 t)] \qquad (1b)$$

Using the definitions[21] $X = k_A + k_{AB}$, $Y = k_B + k_{BA}$, and $Z^2 = k_{BA} k_{AB}$, 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. For the case where B is dark only $A(t)$ contributes to the fluorescence decay. 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 constants and not on any ground-state

equilibrium. $(k_1 + k_2)/2 = (X + Y)/2$ is the average rate of decay of the two species, A and B, including the interchange processes. The separation of the two decay rates $k_1 - k_2 = (1/2)[(X - Y)^2 + 4Z^2]^{1/2}$ is always greater than $(X - Y)$, the separation of the decay rates of the two species. If $(X - Y)^2 \gg Z^2$, the decay rates become X and Y and $\alpha = 0$ (or 1 depending on the sign of $X - Y$).

The usual mechanism by which an excited state can be nonfluorescent is to decay rapidly via a radiationless decay. However, this precludes the reverse reaction necessary for a double-exponential decay if B is dark. 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. To figure in this kinetic scheme this species must be formed from A on a ns time scale. We propose the tryptophanyl radical as this dark intermediate species. This species is formed by very rapid (femtosecond to picosecond) ionization to form the contact radical ion pair and then proton transfer to a basic species. In this case the abstract kinetic scheme above becomes Scheme 2

SCHEME 2

$$\begin{array}{ccc} \mathbf{WH^*} + \mathbf{Q} \rightleftarrows [(\mathbf{WH^{+\cdot}})(\mathbf{Q^{-\cdot}})] \rightleftarrows [\mathbf{W^{\cdot}} + \mathbf{HQ^{\cdot}}] \\ \downarrow \qquad\qquad\qquad\qquad\qquad \downarrow \\ \mathbf{WH} \qquad\qquad\qquad\qquad\qquad \mathbf{X} \end{array}$$

where WH and WH* are the ground and excited states of tryptophan with the indole N−H hydrogen indicated explicitly, Q is a collisional electron acceptor, and X is either the ground state of tryptophan (i.e., WH), a triplet state of the radical pair, the triplet state of tryptophan or other species. $WH^{\bullet+}$ is the radical cation of tryptophan, and $Q^{\bullet-}$ is the radical anion of the species accepting the electron. The consecutive forward process $W^* + Q \rightarrow [(W^{\bullet+})(Q^{\bullet-})] \rightarrow [W^{\bullet} + HQ^{\bullet}] \rightarrow X$ leads to quenching of the excitation. Electron transfer is the usual mechanism of fluorescence quenching as discussed below. The proton-transfer step is shown as occurring to the transient radical anion, but since the pK_a of the radical cation of trytophan is 4.2, there are other bases that could act as the acceptor. The importance of hydrogen bonding between the indole N−H and the electron and proton acceptor is discussed below.

In all that follows we will assume the electron-transfer steps are very fast and that the proton-transfer steps are rate limiting. Then Scheme 2 becomes equivalent to Scheme 1 with $K_{GX} = 0$ (or, if X = G, $K_{GX} = 1$). The details of this model are not relevant to the following kinetic argument. A reversible process that involves a dark intermediate and which occurs on the ns time scale is all that is required.

Application to Data for Proteins in the Absence of External Quenching Agents

To illustrate the applicability of this kinetic scheme in the case of tryptophan fluorescence, we take the specific case of a variant of bacteriophage T4 lysozyme in which the only tryptophan residue present is at the buried location 138.[17,19] Residue 105 in this variant is a glutamine as in the wild type. As discussed below, it is believed that this residue quenches the fluorescence of tryptophan 138. The decay of the fluorescence of this protein at several excitation wavelengths is shown in Figure 1. The decays are clearly non-single-exponential. The are also independent of wavelength. This last point demonstrates that if ground-state heterogeneity is present, the two species in

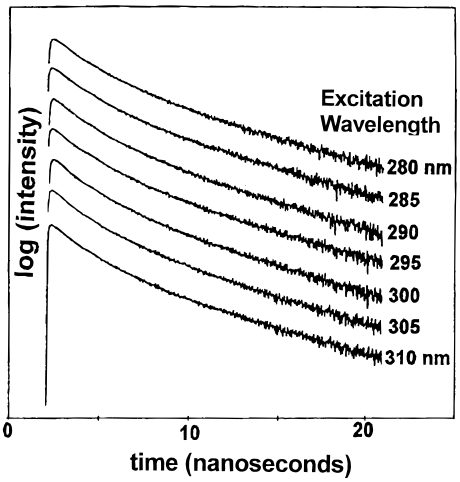

Figure 1. Logarithmic fluorescence decay curves of bacteriophage T4 "YWY" (105Q) in which tryptophan residues 126 and 158 have been replaced by tyrosine, leaving only tryptophan residue 138. The excitation wavelengths are indicated. When these data are subtracted in pairs and plotted as modified residuals, it is clear that they are all identical.

equilibrium have the same absorption spectra. 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} and $1 - \alpha = 0.21$, $k_2 = 1/3.1$ ns $= 0.32$ ns^{-1}. From some additional data to be discussed below[22] we deduce that the fundamental rate constant k_A, the decay rate of W* in the absence of quenching, is 0.2 ns^{-1}. From this we can determine that[21] k_{AB}, the rate of the forward process, is 0.6 ns^{-1}, k_{BA}, the rate of the reverse process, is 0.1 ns^{-1}, and the rate of decay of the radical pair, k_B, is 0.35 ns^{-1}.

This analysis shows that reasonable rate constants are obtained from an analysis of this decay data. As expected, the forward and reverse rate constants are on the order of the decay constants. This ensures a double-exponential decay with appreciable amplitudes and distinct lifetimes. The relatively rapid forward processes results in overall quenching of the fluorescence. The reverse rate for electron transfer is one-sixth that of the forward rate. This near unity value for the ratio of these rate constants is essential to the argument since it places the radical pair at an energy that is similar to the excited state of tryptophan, permitting recombination luminescence. In the specific model presented here the free energy difference between the radical pair, $[W^{\bullet} + HQ^{\bullet}]$, and the initial excited state, WH* + Q, has two components. One is the electron-transfer step, and the other is the proton-transfer step. The oxidation potential of tryptophan is 1.25 V, but the reduction potential of glutamine is unknown. Similarly, the pK_a of the radical cation is 4.2 but the pK_a of the accepting base is unknown. It is possible that in the presence of a sufficiently strong base, an unfavorable electron-transfer process may occur by coupling to the proton-transfer step. Conversely, it is possible that if a stable radical ion pair is formed, then in the absence of a suitable adjacent base, the proton-transfer step may not occur on the ns time scale. This description thus includes both limits of electron- and proton-transfer quenching.[23] It should be noted that the glutamine radical anion, of unknown basisity, is a likely candidate for the initial proton recipient on kinetic grounds simply because glutamine-105 is hydrogen bonded to the indole N−H of tryptophan-138 (Figure 2).

Many amino acids are known to cause collisional quenching of the fluorescence of tryptophan in aqueous solution.[23,24] One of the moderate quenchers of tryptophan fluorescence is

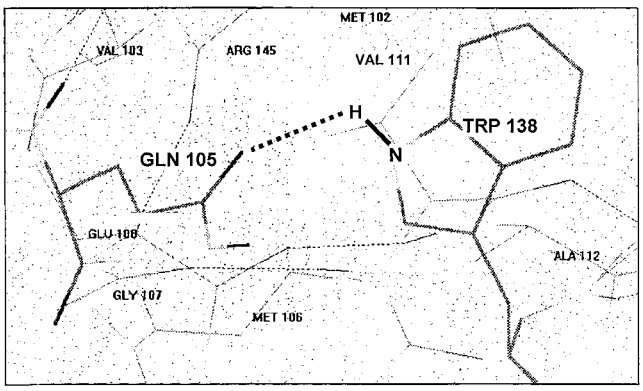

Figure 2. Structure of bacteriophage T4 lysozyme in the vicinity of tryptophan 138, showing glutamine 105 to which the indole N−H of tryptophan 138 is hydrogen bonded.

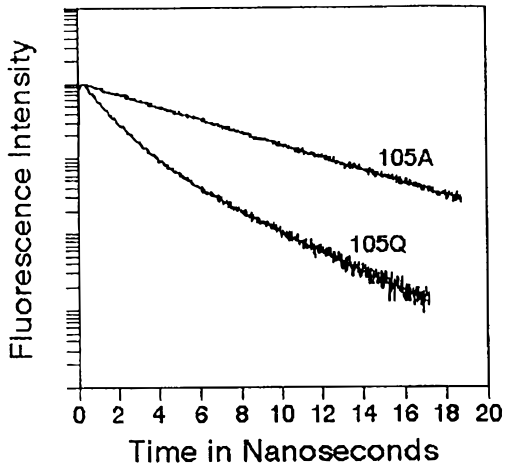

Figure 3. Logarithmic fluorescence decay of bacteriophage T4 lysozyme "YWY" (see legend to Figure 1) with glutamine at position 105 ("105Q") and YWY/Q105A with alanine at position 105 ("105A"). Reproduced with permission from Plenum, 1994.

glutamine. For this reason we decided to change the hydrogen-bonded glutamine-105 residue to an alanine.[17] The resulting protein exhibited a 3-fold increase in fluorescence intensity, consistent with the lower quenching efficiency of alanine relative to glutamine. The time dependence of the fluorescence of this mutant protein is compared to that of the protein with a glutamine residue at position 105 in Figure 3. The decay of the fluorescence of the variant of T4 lysozyme that has alanine at position 105 instead of glutamine is essentially a single-exponential decay with a lifetime of 5.1 ns.[17]

This glutamine-105 to alanine amino acid replacement results in no changes in the structure of the protein other than the specific amino acid side chain atoms.[25] Replacement of glutamine-105 by histidine results in a *more* complex (and pH dependent) fluorescence decay,[26] and Q105S with serine at position 105 has a non-single-exponential decay behavior that is more complex than that of the Q105A variant but not as complex as that of the histidine variant. These results are all consistent with the quenching order observed for amino acids[23,24] and with the observation that tryptophan residues that are highly quenched often exhibit a complex time dependence in their fluorescence decay behavior.[27] This result and prior observations 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.

Observations Supporting the Mechanism. Collisional quenching of fluorescence is often interpreted in terms of electron-transfer process.[23,28] The values observed for amino acid derivatives[23,24] span more than 1 order of magnitude. There is a strong (>0.95) correlation 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 in 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.[29]

Tryptophan and its analogues are known to photoionize in aqueous solution.[5,30] The ionization quantum yield is 8% with excitation at 280 nm. 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. Recent studies carried out with 200 femtosecond temporal resolution indicate that the instantaneous yield of electrons with excitation at 300 nm is on the order 40−60%. It is also known that much of the photochemistry of tryptophan-containing peptides can be understood in terms of radical reactions.[31]

The phenomenon of recombination luminescence is well established for aromatic organic species.[32,33] This process is usually described for fluorescent organic compounds in solutions at low temperature ("glasses"). The mechanism is photoejection of electrons into the matrix where they become trapped in low-energy sites. Recombination luminescence is associated with either thawing the glass or illumination with infrared radiation in the region of absorption of solvated electrons.

Another series of observations that appear to be relevant to this hypothesized mechanism concern the triplet state of tryptophan. Space does not permit a discussion of these results except to note that the phosphorescence of tryptophan in aqueous solution at room temperature can be quenched by amino acids,[34] the decay kinetics observed for the triplet state of tryptophan from room-temperature phosphorescence is almost always distinct from that observed via transient absorption measurements,[35] and the decay of the room-temperature phosphorescence of proteins is also multiexponential.[36] All of these results are consistent with an electron-transfer quenching mechanism with radical intermediates for the quenching of the triplet state of tryptophan.

Addition of an External Quenching Agent. According to the multiple conformational state explanation of the heterogeneity of tryptophan fluorescence, the ratio $\alpha/(1-\alpha)$, is equal to the equilibrium constant K_{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 $X = k_A + k_{AB}$ and k_1 and k_2 depend in a complex way on all the rate constants.

Suppose that an extrinsic quencher, e.g., acrylamide, is added to a solution of a protein-containing tryptophan. In the multiple conformational state model, each conformational species will have its lifetime shortened (perhaps in distinct ways) but, unless there is binding of the quencher to the protein and that binding is preferential to one conformation over another, the equilibrium ratio $\alpha/(1-\alpha)$ will remain constant.[2] In contrast, in the reversible dark state model the ratio $\alpha/(1-\alpha)$ will, in general, vary with addition of quencher. This is because the rate of decay of the initially excited state, k_A, will increase with added quencher, increasing the amplitude of the short-lived component.

The best case for such a study is one in which the amplitude of the short-lived component in the absence of quencher is small. This permits the largest variation in $\alpha/(1-\alpha)$ with added

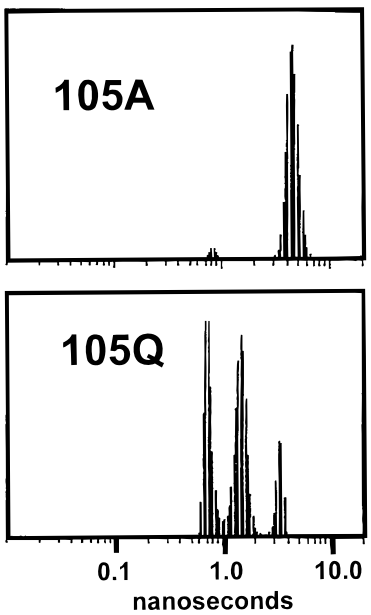

Figure 4. Maximum entropy method analysis of the fluorescence decay data for 105A and 105Q variants of T4 lysozyme. Excitation 300 nm; $T = 20$ °C.

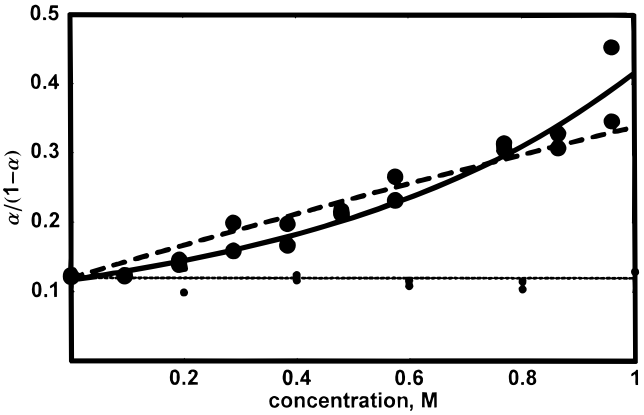

Figure 5. Variation in the ratio $\alpha/(1 - \alpha)$ for bacteriophage T4 "YWY"/Q105A with added acrylamide. There are two data points for each concentration, some of which overlap. The solid line is the optimized fit of a Stern−Volmer treatment of the quenching as applied to the reversible dark state model with $\Delta_q = 0.65\ M^{-1}\ s^{-1}$. The dashed line is a fit of a differential binding model with a value of $K_A/K_B = 20$ with species A having the shorter lifetime. The horizontal dashed line through the smaller circles refers to the results for propanamide.

quencher. The 105A variant of T4 lysozyme has a very small short lifetime component, as revealed by the maximum entropy method analysis[37−43] (Figure 4). The amplitude of this short-lifetime component ranges in magnitude from 3 to 12% depending on conditions.

To explain the small short-lifetime component of the decay of the fluroescence of the 105A variant of T4 lysozyme, it is necessary to postulate the presence of one or more electron acceptors in the vicinity of trp-138. The specific nature of the acceptor species is not known in this case but there are numerous possibilities including the peptide bond.[44] The fit of a double-exponential form to this decay data for the conditions used in Figure 5 gives $\alpha = 0.1$, $k_1 = 1.33\ ns^{-1}$, $k_2 = 0.22\ ns^{-1}$. From this we obtain[21] $X_0 = 0.33\ ns^{-1}$, $Y_0 = 1.22\ ns^{-1}$, and $Z^2 = 0.11\ ns^{-2}$. Since $(X - Y)_0^2 \gg Z^2$ for this case, $k_1 \cong X_0$ and $k_2 \cong Y_0$.

To decompose X_0, Y_0, and Z^2 for the 105A variant into their elemental components, a value of k_{A0} must be assumed. A reasonable value is $k_{A0} = 0.2\ ns^{-1}$. This yields the individual rates $k_{AB} = 0.131\ ns^{-1}$, $k_{BA} = 0.84\ ns^{-1}$, and $k_B = 0.38\ ns^{-1}$. This set of parameters implies that the radiationless decay of the contact radical ion pair A to the ground state is roughly twice as fast as the decay of the excited singlet state of tryptophan. The hypothesized electron- and proton-transfer process in the forward direction is slower than the rate of decay of the excited state; the reverse process is about 6 times faster than the forward process. Electron plus proton transfer is not very favorable at equilibrium in this case. Again this may be due to a less favorable free energy due to either the electron-transfer step or the proton-transfer step.

The variation of $\alpha/(1 - \alpha)$ as a function of acrylamide concentration for the 105A variant of T4 lysozyme is shown in Figure 5. This quantity is clearly not constant for this series of experimental results. This is inconsistent with the multiple conformational state hypothesis. 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, WH* or A, and the radical pair, $[(W^{\bullet})(HQ^{\bullet})]$ or B. This quenching can presumably be described in the usual pseudo-first-order (Stern−Volmer) fashion: $k_A = k_{A0} + k_{qA}[Q]$, where [Q] is the concentration of collisional quencher with bimolecular quenching constant k_{qA}. This causes k_A to increase linearly with added quencher concentration. $X = k_A + k_{AB} = k_{A0} + k_{AB} + k_{qA}[Q]$ therefore also increases linearly with [Q]. Similarly, $Y = k_{B0} + k_{BA} + k_{qB}[Q]$. If $k_{qA} = k_{qB}$ then $X - Y$ is independent of [Q] and $\alpha/(1 - \alpha)$ is constant. In general, $X - Y = (X - Y)_0 + \Delta_q[Q]$, where $\Delta_q = k_{qA} - k_{qB}$ is the difference in the collisional quenching rate constants for the two species. The expression for $\alpha/(1 - \alpha)$ involves X_0, Y_0, and Z^2 (all of which can be determined from the $[Q] = 0$ data) and the one new parameter Δ_q. This quantity is adjusted to produce the smooth curve in Figure 4. The degree of agreement shows that these data are consistent with the ionization/recombination hypothesis.

It might be possible to reconcile these quenching observations with the multiple conformational state hypothesis by arguing that differential binding of acrylamide might occur to the protein conformational species with the shorter lifetime, shifting the equilibrium toward that species. Equal binding to both species will, of course, not shift the equilibrium. Previous studies of other proteins indicate that binding of acrylamide is very weak.[45] The dashed curve shown in Figure 4 results from an optimized fit of a differential binding model to the data. The value of the ratio of the binding constant for binding to the short-lived species to that of the long-lived species has to be 20 in order to obtain this fit. Such a large ratio seems highly unreasonable for such nonspecific binding. A further test of this possibility is provided by an experiment in which propanamide, a close structural analogue of acrylamide, is added in place of acrylamide. No quenching is observed. The points and horizontal line shown at the bottom of Figure 4 show that there is no change in the amplitudes of the decay components. This further indicates that a differential binding mechanism is not operative.

An alternative possibility is that there are two conformational species present and that acrylamide binds more or less equally and weakly to both conformations but that only one species, the long-lived conformation, is so strongly quenched as to be unobserved in the time-resolved fluorescence. The short-lived conformation must be unaffected in its decay by this binding. To increase the value of $\alpha/(1 - \alpha)$ from 0.1 to 0.25, as is observed, would require that approximately two-thirds of the long-lived species fluorescence be lost from the decay. This possibility can be eliminated by examination of the initial

absolute intensity of the fluorescence decays for this series of experiments. These values are all the same.

Multiple Acceptors and Multiple Decay Components. There are many cases where the fluorescence decay of tryptophan in proteins is clearly described by more than two exponential components.[19,24−27] In our experience this appears to be the case when a tryptophan residue is highly quenched.[27] A more detailed analysis of the decay data of the 105Q variant (using a maximum entropy method; Figure 4) indicates that there are three components to the decay. The lifetimes are 0.65, 1.4, and 3.3 ns with amplitudes 0.40, 0.45, and 0.15. The two-component analysis described above merges the two short lifetime components, which differ by only a factor 2 in lifetime. The form of the decay is thus

$$I(t) = 0.40\exp(-1.54t) + 0.45\exp(-0.71t) + 0.15\exp(-0.30t)$$

We analyze this case under the assumption that the 105Q case consists of whatever (unknown) transfer process that occurs in 105A plus the much more efficient glutamine-105 transfer case.

The generalization of the model given above that is necessary to describe this situation is Scheme 3 where A = WH* and B

SCHEME 3

$$\begin{array}{ccccc} \mathbf{B} & \rightleftarrows & \mathbf{A} & \rightleftarrows & \mathbf{C} \\ \downarrow & & \downarrow & & \downarrow \\ \mathbf{H} & & \mathbf{G} & & \mathbf{J} \end{array}$$

and C are the radical pairs formed by transfer of an electron and a proton to either of two distinct acceptor species. Since the proton-transfer step is postulated to be rate limiting, what is necessary is that there be two places for the proton to go. This might or might not involve two distinct electron-transfer acceptors. This detail is not relevant to the general argument. The full 3 × 3 kinetic rate matrix for this case involves nine independent parameters, the seven indicated by arrows in Scheme 3 plus the two associated with the interconversion process B ⇌ C corresponding to the interchange of an electron and a proton from one radical species to another. These are, however, constrained by the requirement that the equilibrium constant for an A ⇄ B ⇄ C process must be unity. At present we ignore this B ⇌ C possibility, leaving seven parameters.

The physical reality underlying this model greatly simplifies the situation, however, because we have already analyzed the left-hand side of this reaction scheme (the upper left 2 × 2 of the rate constant matrix). So if there is any correspondence between the molecules being studied and the mathematics used, the triple-exponential decay involves only three new parameters, those associated with the A ⇄ C interconversion and the decay C → J. The other four parameters are fixed at the values determined from the double-exponential analysis of the 105A variant. The experiment gives five numbers (three decay times and two relative amplitudes) to be fit with these three parameters. Numerical diagonalization of the asymmetric 3 × 3 rate constant matrix for various parameter values gives a reasonable fit with $k_{ac} = 0.65$ ns^{-1}, $k_{ca} = 0.09$ ns^{-1}, and $k_c = 0.26$ ns^{-1}. These values are *very* close to those obtained when the same data were fit as a double-exponential decay and analyzed in terms of only one electron exchange process. This is what one would expect since the small component in the 105A case is *almost* negligible and the two decay components of the 105Q case have very similar lifetimes and so can *almost* be added together. This also indicates that the inversion process is numerically stable. The resulting triple exponential decay is

$$I(t) = 0.36\exp(-1.47t) + 0.51\exp(-0.82t) + 0.13\exp(-0.25t)$$

The individual parameters are within realistic experimental error estimates equal to the parameters extracted from the fluorescence data. Addition of the BC interchange term (in conjunction with the restriction imposed by microscopic reversibility) decreases the degree of agreement between experiment and the model in this case.

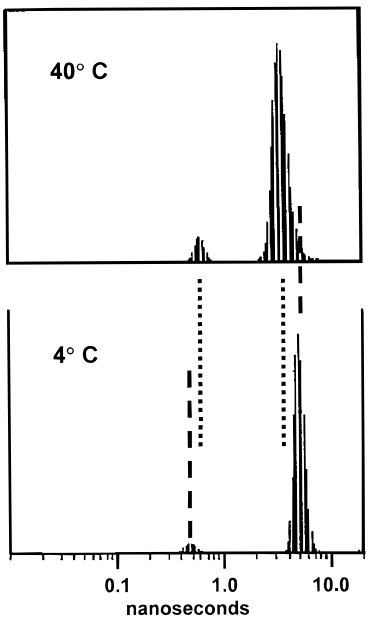

Figure 6. Maximum entropy analysis of 105A variant of T4 lysozyme at 40 and 4 °C.

Discussion

A general prediction of the kinetic scheme presented above is that as the temperature is raised and the forward and reverse rates k_{AB} and k_{BA} increase there should be a merging of the two relaxation rates in the way NMR chemical shifts merge as the exchange becomes rapid. This is not so easy to see in complex multicomponent decays but is clearly seen in the case of the 105A variant of T4 lysozyme, as shown in Figure 6. Data and analyses of this type have been performed for the variant of T4 lysozyme at temperatures of 4, 10, 20, 30, 40, 50, 60, and 70 °C. At 60 and 70 °C three peaks appear. T_m for this protein is 59.2 °C.[17] The data for the six lower temperatures all obey the trend illustrated in Figure 6.

The simplest description of the overall process we are proposing for the photophysics of tryptophan in proteins begins with optical excitation to a state that has an expanded orbital extent involving significant overlap with neighboring groups. Ionization may be considered to occur on a nearly instantaneous basis. Most of the time recombination follows rapidly to form the excited electronic state. If there is a group in the neighboring region that can accept an electron, then this recombination process is inhibited and a contact radical ion pair is formed. In most cases this results in back electron transfer, often on the ground-state surface, and thus quenching of the fluorescence. If, however, the indole N−H can transfer a proton to a neighboring base, which might be the radical anion, then the neutral radical pair is formed. This has a lifetime on the

order of a few ns (2 ns for the trp-138/gln-105 case). To stabilize the radical pair on the ns time scale, it may be necessary that the indole N−H be hydrogen bonded to the base. In this case the entire process can be thought of as an excited-state tautomerization reaction of the super-molecule formed by the tryptophan/quenching base pair. The proton-transfer step in that case may involve tunneling of the indole N−H proton. It is interesting to note in this respect that one of the few cases of single-exponential decay behavior for a protein is apoazurin where the indole chromophore of tryptophan is apparently not involved in any hydrogen bond.[46]

This hypothesized mechanism predicts that there will be an effect of indole N−H deuterium substitution on the fluorescence quantum yield as is observed.[23,47] It also predicts that there will be an effect of indole N−H deuterium substitution on the amplitudes of the decay components an effect that has so far not been reported.

The generality of this proposed mechanism cannot be established with a study of a single protein. On the other hand, almost all amino acid side chains are more or less effective quenchers[23,24] and thus it is hard to see how the steps operative in the present mechanism can be completely absent in other cases.

The emission properties of the 105Q variant of T4 lysozyme are almost independent of emission wavelength.[19] When the data are analyzed as a sum of three exponential terms, the average lifetime varies from 1.3 ns at 340 nm to 1.6 ns at 400 nm, with the major origin of the increase in lifetime being due to an increase in the lifetime of the predominant middle lifetime component and an increase in the amplitude of the minor long-lived component. Most proteins show a larger variation in their decay properties, but the variation is usually in the same sense, i.e., longer average decay at longer emission wavelength. The model presented above does not explicitly contain any features that are relevant to this aspect of protein tryptophan photophysics. One way of incorporating this behavior into the present model is, of course, to retain the aspect of previous "relaxational" descriptions of tryptophan photophysics. In this view the red shift follows from relaxation of the tryptophan surroundings following electronic excitation stabilizing the new excited state dipole moment. If this relaxation process is viewed as a shift of the entire emission envelope to lower energy with time, then it necessarily leads to a temporal behavior in the red edge of the emission that rises in time before it decays. This behavior is easily observed for the time dependence of the fluorescence of tryptophan in viscous polar solvents, but it has not been conclusively observed for a protein with a single tryptophan residue.

A variant of this behavior more directly related to the present model is based on the process designated "radiative recombination" (as distinct from "recombination luminescence"). Radiative recombination[33] is the process in which a contact radical ion pair undergoes emission of a photon and simultaneous electron transfer. If the two components of the ion pair (in this case the indole chromophore and some quencher such as glutamine) are viewed as a "super molecule", then radiative recombination is simply fluorescence from an excited state of the super molecule that involves a very large dipole moment. Time dependent stabilization of the contact radical ion pair due to relaxation of the surroundings then results in emission wavelength dependence. There will still be the same objection that such emission should produce a rise in intensity in the red edge of the spectrum. It is possible that the combination of a small radiative emission component and a distinct Franck−Condon spectral envelope will cause the emission from this relaxational component to remain submerged under the main decay due to direct emission and recombination luminescence.

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(22) A double-exponential fluorescence decay 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 105A 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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