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Histidine-tryptophan interactions in T4 lysozyme: 'Anomalous' pH dependence of fluorescence

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Abstract

A variant of T4 lysozyme which contains only a single tryptophan residue (at position 138) has been prepared (W126Y/W158Y designated 'YWY'). Two additional mutations to YWY have been prepared involving replacement of glutamine 105, which hydrogen bonds to the indole N–H of trp 138 in wild type, with either a histidine (YWY/Q105H) or an alanine (YWY/Q105A). The fluorescence properties of these two species are investigated as a function of pH. YWY/Q105A exhibits essentially a single exponential fluorescence decay (5% $\tau = 0.35$ ns 95% $\tau = 5$ ns) and almost no pH dependence in steady state or time resolved fluorescence behavior. In contrast, YWY/Q105H exhibits complex fluorescence decay over the entire pH range used in these experiments. As the pH is lowered from 8 to 4, there is an increase in the quantum yield and a change in the average lifetime (from 2.0 to 3.1 ns). Using this data, the pK_a of histidine 105 has been determined to be 5.9. These results are contrasted to those from other proteins which show a pH dependent tryptophan fluorescence associated with a neighboring histidine or other residue. Quenching behavior in terms of the stereochemistry of the tryptophan-histidine interaction and implications of these results for current models of complex fluorescence behavior of single tryptophan proteins are also discussed.

Keywords: Tryptophan; Fluorescence; Histidine; Lysozyme

1. Introduction

The presence of a tryptophan residue in a protein provides a potentially valuable experimental probe. Although studies which measure changes in tryptophan fluorescence quantum yield and/or energy transfer have proven valuable for the study of protein structure and ligand binding, the complex behavior of tryptophan fluorescence, including lifetime and anisotropy, is still not well understood ([1,2]). Resolving these complexities may provide even more valuable information about protein structure and dynamics. The photophysical behavior of tryptophan residues in proteins is largely attributed to interactions with neighboring residues in the folded protein. By mutating amino acid residues adjacent to trp 138 in T4 lysozyme, we have previously shown ([3–6]) that effects of these substitutions on lysozyme fluorescence can yield information about how neighboring amino acids change the photophysical properties of this tryptophan residue. The crystal structure of wild-type lysozyme shows that the indole N–H of trp 138 is hydrogen bonded to gln 105 ([7]). It was found that this glutamine was responsible for

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quenching the fluorescence of trp 138 ([2,8]). Substitution of glutamine at position 105 by alanine, valine, isoleucine and glutamic acid have significant effects on trp 138 fluorescence. The direction of these effects correlates well with the ability of these residues to quench tryptophan fluorescence in solution ([9]). The results of the substitutions were complicated by the fluorescence of the two distant tryptophans because these mutants were constructed in the wild-type lysozyme background which contains three tryptophans. Harris and Hudson also examined a lysozyme mutant in which two of the tryptophans in T4 lysozyme have been changed to tyrosines (W126Y/W158Y), this mutant, termed YWY, leaves a single tryptophan at position 138 that can be examined without the complication of multiple tryptophan emission ([5]). Despite removal of trp 126 and trp 158, trp 138 still exhibits complex fluorescent behavior, most likely attributed to interactions with gln 105. Recently, we have shown that substitution of gln 105 with alanine in YWY (YWY/Q105A) results in tryptophan fluorescence with a single exponential decay ([2]). These results are significant because they demonstrate that the complex behavior of tryptophan fluorescence in YWY could be simplified by removing the interacting residue at position 105. The amino acid at position 105 is therefore, in some fashion, the origin of the complex fluorescence decay behavior behavior of trp 138. This system can be used to substitute different residues at position 105 in YWY in order to specifically isolate their effects on trp 138 fluorescence. In this article, we examine the effect of placing a histidine residue at position 105 in YWY (YWY/Q105H).

It has previously been observed that protonation of histidine residues results in changes in the photophysics of adjacent tryptophans in proteins ([10–13]). Solution studies have shown that the protonated form of histidine is a more efficient quencher of tryptophan in solution than the neutral form ([9,10]). The most extensive study of pH dependent fluorescence of a tryptophan residue near a histidine in proteins has been carried out for barnase ([11,12,14]). Protonation of his 18 of barnase results in a 3-fold decrease in the quantum yield, and a 2-fold decrease in the lifetime of the adjacent trp 94. The pK_a of this histidine 18 of barnase was determined to be 7.75. In our experiments, we examined the pH dependence of trp 138 fluorescence in YWY with a histidine at position 105. We were able to attribute the effect of pH on fluorescence specifically to this histidine residue by comparing the results to a similar titration with YWY/Q105A. The results are substantially different from the observations for barnase and other proteins, since the quantum yield and lifetime for trp 138 of T4 lysozyme YWY/Q105H increase at low pH rather than decrease.

2. Materials and methods

2.1. Mutagenesis

YWY lysozyme was obtained from Lawrence McIntosh and Cynthia Phillips, Institute of Molecular Biology, University of Oregon. The YWY gene was used as a template to prepare Q105A and Q105H by site-directed mutagenesis ([15]).

2.2. Protein purification

The genes for all mutants were cloned into the vector pCw. The plasmids were used to transform *E. coli* strain K38. Protein production was induced by IPTG. The lysozymes were purified by cation exchange chromatography ([16]). Protein purity was shown by SDS gel electrophoresis. Previous studies have shown the YWY mutant to have near 100% activity of the wild-type (Lawrence McIntosh, unpublished results). All the mutants in this study have over 60% of wild-type activity.

2.3. Steady state fluorescence

Concentrated stocks of Q105A and Q105H around 25–30 mg ml^{-1} were prepared. Samples were prepared for titration as follows: Sample buffer (0.1 M $NaPO_4$, 0.1 M NaCl, 0.01 mM 2-mercaptoethanol) was adjusted to the desired pH using concentrated HCl and NaOH. The ionic strength was kept constant at 0.2 M by the addition of an appropriate amount of NaCl. 20 μL of protein from the concentrated stock was injected into 1 ml of the sample buffer prepared at the appropriate pH. This was sufficient protein to give an absorbance of 0.08 at 300 nm. Samples were mixed by inversion to avoid protein adhesion to the

cuvette. Steady state fluorescence spectra were obtained with a SLM-8000 fluorometer (SLM industries, Urbana, IL). All measurements were performed at room temperature with excitation at 300 nm in order to isolate tryptophan fluorescence. Two spectra were taken for each sample to insure good mixing and titrations were carried out in random order to avoid any systematic error leading to false trends.

2.4. Time resolved fluorescence

The titrations were performed in the same way as the steady state experiments. Measurements were obtained by time-correlated single photon counting,. Detailed descriptions of the instrumentation and data collection are given elsewhere ([17]). The frequency doubled output of a Spectra Physics 3640 mode-locked Nd:YAG laser was used to synchronously pump a rhodamine 6G dye laser cavity dumped at 800 KHz. A Hamamatsu R1564U proximity focused 12 mm microchannel plate was used as a detector. The instrument response was typically 100 ps in width. The excitation wavelength was 300 nm and the emission was collected through 360 nm band pass interference filters. The temperature was kept constant at 20°C. Descriptions of the data analysis procedures have been given previously ([4]).

2.5. Circular dichroism

Samples were prepared in same buffers as above at a protein concentration of 1.6 μM. CD spectra were measured on a JASCO-600 spectrophotometer at 23°C. Thermal unfolding studies were performed using a temperature ramp from 0° to 98°C at a rate of 1°C min^{-1}.

3. Results

3.1. Steady state fluorescence

Fig. 1 shows the comparative fluorescence spectra of YWY/Q105A, YWY/Q105H and YWY at pH 6.1. Relative to the YWY species, the YWY/Q105A mutant has a 3-fold increase in quantum yield and the YWY/Q105H mutant has a 2-fold increase in quantum yield. These data show the wild

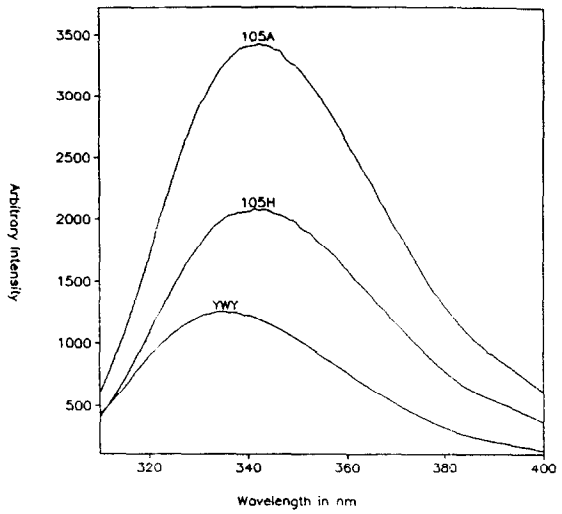

Fig. 1. Comparative fluorescence emission spectra of YWY, YWY/Q105H and YWY/Q105A variants of T4 lysozyme obtained at a pH of 6.1 and a temperature of 23°C. In each case the absorbance of the solutions was 0.08 at the excitation wavelength of 300 nm.

type glutamine to be the most efficient quencher, followed by histidine, and alanine as the least effective. This trend parallels the tendency of the individual amino acids to quench tryptophan fluorescence in solution for histidine in its neutral form (Table 1). The change in fluorescence intensity as a function of pH for the YWY/Q105H variant is shown in Fig.

Table 1
The bimolecular quenching rate constants for the amino acids for quenching the steady state fluorescence of acetyl tryptophan ([9])

		$k(10^9\ M^{-1}\ s^{-1})$
S–S	disulfide	large
H^+	histidineH^+	2.7
C	cysteine	1.8
P	proline	0.6
M	methionine	0.5
Q	glutamine	0.5
N	asparagine	0.4
R	arginine	0.4
H	histidine	0.3
S	serine	0.3
T	threonine	0.2
G	glycine	0.2
K	lysine	0.2
V	valine	0.2
A	alanine	0.1

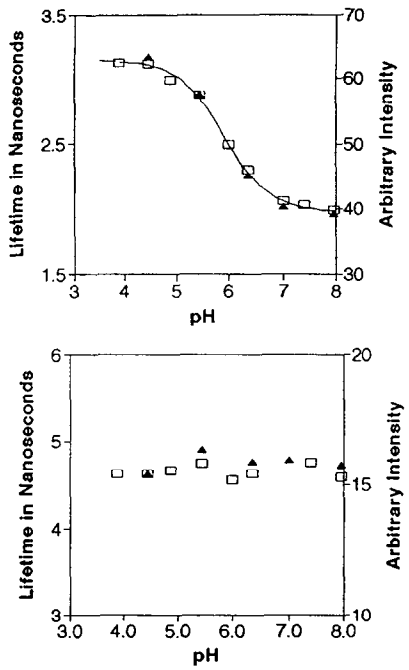

Fig. 2. The pH dependence of the fluorescence of (A) YWY/Q105H and (B) YWY/Q105A variants of T4 lysozyme. (□) fluorescence intensity; (▲) average fluorescence lifetime.

2(a). At pH's below 4, the intensity decreases sharply, presumably due to denaturation. Over the pH range from 4 to 8, the data of Fig. 2(a) can be fit with a calculated curve which yields a pK_a of 5.9. Fig. 2(b) shows the corresponding data for a titration of YWY/Q105A. There is no significant change in fluorescence intensity as a function of pH, clearly implicating the histidine residue as the cause of the pH dependent fluorescence change in the YWY/Q105H mutant. The spectral maximum for the YWY/Q105H mutant is near 340 nm, the same value observed for YWY/Q105A, slightly red shifted from the wild type. The fluorescence maximum is independent of pH.

3.2. Time resolved fluorescence

Fig. 2 also shows the change in average lifetime as a function of pH for YWY/Q105H and YWY/Q105A. For YWY/Q105H this change in average lifetime corresponds to the change in fluorescence intensity and can be fit to the same titration curve. For the YWY/Q105A mutant there is no significant change in the average lifetime, once again implicating the histidine residue as the cause for the titration of YWY/Q105H. The decay curves for YWY/Q105A and YWY/Q105H at pH 4.4 and 7.0 are displayed in Fig. 3. For YWY/Q105H, there is a clear difference in the curves at the two different pHs values with a significantly longer average lifetime at pH 4.4. For the YWY/Q105A mutant the curves for these two pH values are indistinguishable. The parameters obtained from a fit of the sum of exponential components for these curves are displayed in Table 2. In the case of the YWY/Q105H variant, the decays are rather complex requiring at least three exponentials. This is confirmed by a Maximum Entropy Analysis as discussed below. In contrast, the decay for YWY/Q105A is essentially a single exponential over the entire pH range.

3.3. Thermal unfolding

In order to determine if the increase in fluorescence upon titrating the histidine from pH 8 to 4 was

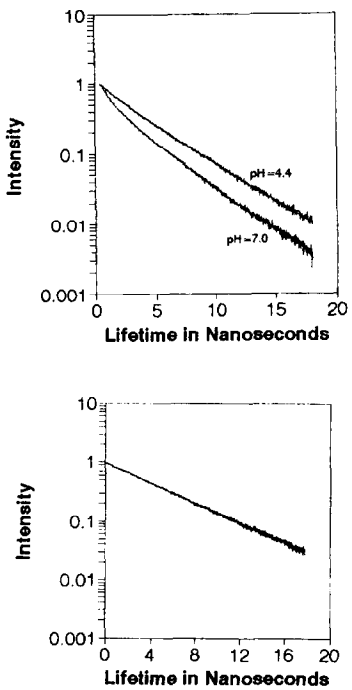

Fig. 3. Fluorescence decay curves (logarithmic) for (A) YWY/Q105H and (B) YWY/Q105A at pH 4.4 and 7.0 (The two curves for YWY/Q105A are indistinguishable.)

Table 2
Parameters obtained from an analysis of fluorescence decay data as a function of pH for YWY/Q105A and YWY/Q105H as a sum of exponential components. All lifetimes in ns

pH	3.1	4.4	5.4	6.4	7.0	7.9
	YWY/Q105A					
α_1/τ_1		0.11/0.26	0.04/0.40	0.05/0.33	0.05/0.38	0.06/0.42
α_2/τ_2	1.00/5.23	0.89/5.17	0.96/5.09	0.95/5.01	0.95/5.01	0.94/5.00
$\langle\tau\rangle$	5.23	4.62	4.91	4.76	4.78	4.72
	YWY/Q105H					
α_1/τ_1	0.16/0.84	0.15/0.48	0.18/0.41	0.26/0.50	0.32/0.48	0.30/0.47
α_2/τ_2	0.59/2.02	0.41/2.71	0.29/2.11	0.32/1.72	0.32/1.68	0.34/1.56
α_3/τ_3	0.25/4.85	0.44/4.48	0.53/4.13	0.42/3.76	0.38/3.64	0.37/3.54
$\langle\tau\rangle$	3.05	3.16	2.88	2.26	2.02	1.97

caused by unfolding caused by introducing a charge into the protein core, circular dichroism spectra and thermal denaturation measurements were performed for YWY/Q105H and YWY/Q105A. The results of the thermal studies show that the YWY/Q105H mutant is slightly less stable than the YWY/Q105A mutant at pH 4.4, but it appears to be folded completely at room temperature even at the acid pH. The values of T_m at pH 4.4 are 59.2°C for YWY/Q105A and 55.0°C for YWY/Q105H. The CD spectra of YWY/Q105H at pH 4.4 and 7.0 are identical. The effects of pH on fluorescence observed in this study are not caused by significant changes in secondary structure.

4. Discussion

4.1. Structure

In wild type T4 lysozyme, gln 105 is hydrogen bonded to the indole N-H of trp 138. Fig. 4 shows a graphical representation of a histidine residue inserted into position 105 using the coordinates of the wild type structure for all other atoms. The histidine residue is positioned so the β and γ carbons are overlaid with the position of the wild-type glutamine. A reasonable fit is observed with no steric crowding. The distance between the histidine N-3 (bearing a lone pair in the normal tautomer) and the indole nitrogen of trp 138 is 3.5 Å without any motion of the indole ring. The motion needed to bring these two groups closer together to form a hydrogen bond seems unrestricted.

4.2. Anomalous pH dependence

The most interesting result of this work is the observation that the fluorescence quantum yield and average lifetime of the YWY/Q105H variant increases as the pH is lowered. This result is opposite in direction, but similar in magnitude, to that observed for a similar titration in barnase ([11]). Other proteins also exhibit this decrease in fluorescence ascribed to protonation of a histidine neighboring a

Fig. 4. Graphical representation of the hypothetical structure of the single amino acid substitution at position 105 in T4 lysozyme. The histidine is positioned so that it overlaps the wild type glutamine as much as possible.

tryptophan residue ([10,18,19]). This behavior observed for YWY/Q105H is also opposite to the effect observed for quenching of tryptophan by histidine in solution where studies as a function of pH have shown that quenching is more efficient for $HisH^+$ than for the neutral form ([9,10,13,20]).

There appears to be some confusion concerning whether the enhanced quenching by protonated histidine is dominated by the fact that it forms a complex with indole and its derivatives in the ground electronic state. Shinitzky and coworkers ([10]) argue in favor of the formation of a B charge transfer complex formed in the ground electronic state between the protonated imidazolium ring compounds and indole derivatives. This complex results in concomitant quenching of the indole fluorescence. Burstein and co-workers ([20]) determine that the protonated form is a much more efficient collisional quencher than the neutral form (by about 6-fold), and present NMR evidence for the stacked nature of the complex. Steiner and Kirby [9], however, show a parallel decrease in the lifetime and steady state intensity for N-acetyltryptophan upon the addition of histidine at pH 5. They argue that the ground state complex formation, while present, is unimportant in quenching. This is also the view of Willaert and Engelborghs [13] as presented in a note revising their earlier conclusions. These authors conclude that the relative efficiency of collisional quenching for the protonated vs. unprotonated form of histidine is about 14. This means that among the amino acids $HisH^+$ is one of the most efficient quenchers of tryptophan fluorescence and His is one of the least efficient.

All of these solution studies agree that histidine quenches tryptophan fluorescence more strongly at low pH. This behavior also has been observed in a variety of studies of peptides and proteins ([10,11,13,14,18]). The increase in fluorescence intensity for the YWY/Q105H mutant of T4 lysozyme is therefore anomalous. This is presumably due to the circumstance that the protonated histidine cannot position itself with respect to the tryptophan ring at the proper distance and orientation the results in the most effective quenching. This suggests that the quenching process is stereo selective, as might be expected. This could arise either because the collisional quenching process, which probably involves electron transfer ([9]), is stereo selective or because a π-complex is needed for quenching and this cannot form in this protein because of steric constraints.

The differences between barnase and T4 lysozyme YWY/Q105H may be explained by the different orientations of the histidine with respect to the neighboring tryptophan. In the case of barnase, the histidinium group is above the six membered ring of the adjacent indole. This is also the case for subtilisin Novo, which, like barnase, exhibits enhanced quenching of a tryptophan residue due to histidine in the protonated form ([19]). In the case of T4 lysozyme YWY/Q105H, his 105 is presumably near the indole N–H with the accepting nitrogen in the plane aromatic ring. It is this difference in position/orientation and the constraints caused by attachment to the protein backbone that is responsible for the difference in the behavior of T4 lysozyme and other proteins including barnase. Another point that must be considered is that protonation of histidine 105 will break the hydrogen bond to the indole N–H. This may result in a conformatinal change in which the quenching histidine group moves further from the indole ring.

We should note that the pH dependence of tryptophan fluorescence in which a higher value of the quantum yield is observed at low pH has been observed in at least one other case. In a study of interleukin 1b [21] observed at about 60% increase in fluorescence as the pH was lowered with a pK_a of 6.59. In this case the quenching at high pH could be eliminated by the addition of 2.5 M NaCl. Site-directed mutagenesis studies and chemical modification indicate that the quenching group is neither histidine nor thiol (cysteine). A carboxylate residue is suggested. However, recent studies ([22]) have shown that the anionic form of a carboxylate residue is a much less effective quencher than is the acid form. Thus, in this case at least, as suggested by the authors, titration of one residue may result in the movement of another residue which is the actual quencher to a position closer to the indole residue.

4.3. Implications for the multicomponent decay of tryptophan fluorescence

The difference in the fluorescence decay behavior between YWY/Q105A (essentially a single expo-

nential) and the multi-exponential decay of YWY/Q105H can be understod as due to the quenching of trp 138 fluorescence by the histidine residue at position 105. This behavior for the analogous case of YWY with glutamine at position 105 has been discussed elsewhere ([2]) in terms of either multiple slowly interconverting states ([1,23–25]) with differential quenching or, alternatively, by a reversible step in the quenching process.

The interpretation of protein fluorescence behavior (including quenching and rotational diffusion behavior) is complicated by the fact that in almost all cases the time dependence of the fluorescence requires a description that involves two or more exponentials ([1]). Such an analysis in terms of discrete exponential for these two species is given in Table 2. These same data sets have been analyzed using the continuous distribution Maximum Entropy method (MEM) ([23,26–31]). The results are shown in Fig. 5. This MEM analysis clearly shows three discrete peaks in the distribution of decay times for the data obtained at pH 7.9, 7.0, 6.4 and 6.0 with constant peak values of 3.35, 1.37, and 0.44 ns and widths of about 0.6, 0.3, 0.03 ns, respectively. The major change with pH in this range appears to be a monotonic decrease in the amplitude of the central (1.37 ns) peak from 27% of the total at pH 7.9 to 17% of the total at pH 6.0. At pH 5.4, near the midpoint of the titration, the central peak moves to 1.96 ns and increases slightly in amplitude. At pH 4.4 the fluorescence decay is well described by a distribution that has only two peaks with maxima at 3.9 and 1.34 ns. This clearly gives the impression that the short lifetime component has been eliminated, but the longer two are unchanged from their values at higher pH.

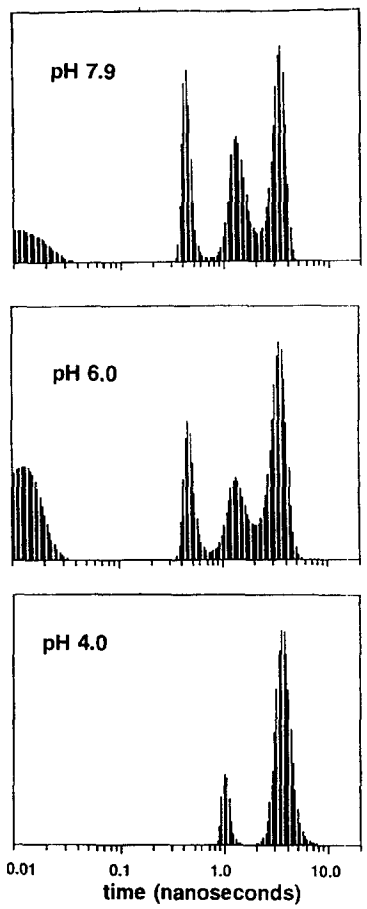

Fig. 5. Maximum entropy analyses of the fluorescence decays of YWY/Q105H at pH; (A) 7.9, (B) 6.0 and (C) 4.4. The intensity at very short times is caused by an artifact of the data collection.

A possible model for these complex, multi-exponential or continuous distributions of decay times has recently been discussed ([2,32]) in terms of a short range collisional quenching process (which may involve electron transfer) resulting in a transient species that can decay by a radiationless path to the ground state. By including the possibility that this quenching process is reversible, the resulting decay of the fluorescence is given by a sum of two exponential terms. The kinetic scheme is shown below.

In this representation the quenching process is presumed to be electron transfer so the intermediate is a contact radical ion pair. This assumption is not crucial to the form of the kinetics. The rate constant k_A includes both the radiative and non-quenching radiationless decay processes for the decay of the fluorescent W^* species. The decay k_B is entirely radiationless. Because of the reversible nature of the quenching process, this scheme yields a double exponential decay in which the amplitudes, as well as the decay rates, are both functions of the rate constants. We postulate that both the forward and reverse rate constants for the quenching process, k_{ab} and k_{ba}, depend on the chemical nature and the ionization state of the quenching species Q, as well as temperature.

We prefer this reversible quenching model in contrast to the more usual multiple conformational state ('rotamer') model ([24,25]) primarily because the replacement of the wild type glutamine residue by an alanine has such a large effect on the complexity of the fluorescence decay ([2]). Glutamine is a much better quencher than alanine (Table 1) and this suggests that there is a connection between the quenching process and the decay heterogeneity. A difficulty with the 'rotamer' or multiple conformational state model is that it order to be applicable the individual conformational states must interconvert slowly on the fluorescence time scale. Molecular modeling and molecular dynamics studies of T4 lysozyme have failed to locate any conformations differing in the local geometry near tryptophan 138 that have lifetimes of more than a few hundreds of picoseconds at most. 'Rotamer' conformers convert to the observed geometry in a few ps.

Further evidence for this reversible quenching model has recently been obtained for the YWY/Q105A protein from the observation that the addition of a soluble quencher (acrylamide) changes the amplitudes of the decay components (manuscript in preparation). The small amplitude short lifetime component becomes the dominant component at high quencher concentrations. This is consistent with the kinetic scheme given above since the amplitudes of the decay components depend on an interaction of all of the rate constants. Addition of a quencher increases the value of k_A by increasing its nonradiative component. This necessarily results in a change in the amplitudes of the decay. A multiple conformational state model would not show any variation in component amplitudes unless there was highly selective binding to one of the conformational states.

In order to describe a decay such as that of YWY/Q105H which is clearly a triple exponential in character, it is necessary to generalize this model to take into account two possible quenching groups, Q_1 and Q_2. In this case one of these groups is the histidine residue at position 105. The other quencher has not yet been identified but it is presumably the same group that causes the decay of YWY/Q105A to deviate from strictly single exponential behavior. The development of this interpretation as it applies to both this case and other mutants of T4 lysozyme will be the subject of a future article.

Acknowledgements

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