Quenching Interactions and Nonexponential Decay: Tryptophan 138 of Bacteriophage T4 Lysozyme

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Site-directed mutagenesis has been used to prepare variants of bacteriophage T4 lysozyme that contain only one tryptophan residue at position 138 and to change the residues in the immediate environment of this buried residue. Replacement of glutamine-105 by alanine results in a 2.7-fold increase in fluorescence quantum yield and converts the fluorescence decay from a highly nonexponential form to a single-exponential decay. This is attributed to electron transfer quenching of tryptophan-138 fluorescence by glutamine-105. Replacement of alanine-146 by threonine results in a 1.6-fold decrease in fluorescence intensity, indicating enhanced quenching by glutamine-105; replacement of glutamine-105 by alanine in this species results in a 5-fold increase in fluorescence intensity. The interpretation of the nonexponential decay of the glutamine-105-containing species is discussed in terms of reversibility of the quenching process.

INTRODUCTION

Bacteriophage T4 lysozyme has proven to be a useful system for exploring the relationship between protein structure and fluorescence properties [1–6]. The three individual tryptophan residues of the wild-type protein (Fig. 1) have been investigated in isolation in studies involving tryptophan to tyrosine substitutions [4]. In three recent studies [1–3] mutational changes have been made to residues proximal to tryptophan 138 and the effects on the fluorescence of lysozyme determined. The substitutions investigated involve changes at position 105 and 146. In the wild-type enzyme position 105 is a glutamine residue which is hydrogen bonded to the indole N–H of tryptophan-138 [7,8]. This puts glutamine in position to quench the fluorescence of tryptophan 138. Replacement of glutamine-105 by other amino acids alters these quenching properties [1–3]. At position 146, replacement of the wild-type alanine with a larger residue, threonine, results in displacement [8] and enhanced

motion of the adjacent tryptophan-138 residue [1–3] indicated by spectral, isotope exchange rate, and differences in susceptibility to enzymatic digestion.

These previous studies of the effects of mutations at positions 105 and 146 on the fluorescence of T4 lysozyme have been carried out in the wild-type background, which contains all three tryptophan residues. In the present study we present the first results in which the background tryptophan residues at positions 126 and 158 have been replaced by tyrosine so that the influence of substitutions adjacent to tryptophan 138 on this residue can be fully revealed.

METHODS

Mutagenesis

T4 lysozyme with a single tryptophan at position 138 was obtained from Lawrence McIntosh and Cynthia Phillips, Institute of Molecular Biology, University of Oregon. The gene for this protein was used as a template to prepare the additional mutants by site-directed mutagenesis according to the method of Kunkel *et al.* [9].

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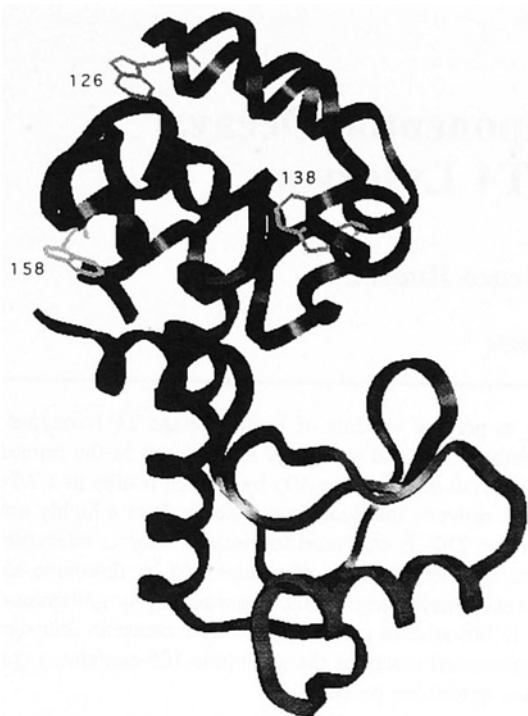[2] To whom correspondence should be addressed.

Fig. 1. The ribbon structure of bacteriophage T4 lysozyme with the three tryptophan residues of the wild-type enzyme indicated.
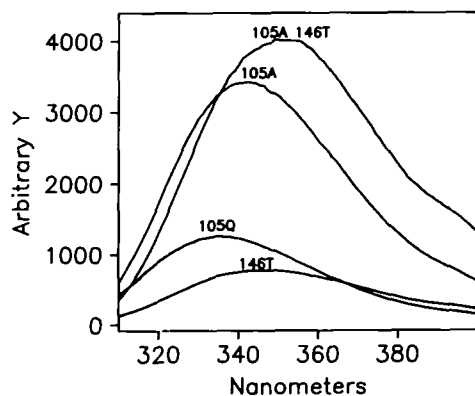

Fig. 2. Fluorescence spectra of T4 lysozyme variants with substitutions at positions 105 and 146. In each case tryptophans at 126 and 158 have been replaced by tyrosine. 105Q is the wild-type species; 105A has alanine at position 105 (and retains alanine at position 146); 146T has threonine at position 146 (and glutamine at 105); 105A 146T has alanine at 105 and threonine at 146.

Protein Purification

The genes for all mutants were placed in the vector pCw. The plasmids were used to transform *Escherichia coli* strain K38. The transformed K38 was grown and protein production was induced by IPTG. The lyso-

zymes were purified by cation exchange chromatography [10]. Protein purity was shown using SDS gel electrophoresis. Previous studies [11] have shown the single tryptophan mutant to have near 100% the activity of the wild type. All the mutants in this study have activity over 50% of that of the single tryptophan mutant, except for A146T/G105A, which showed under 50% activity.

Sample Preparation

Samples with an absorbance of 0.08 at 300 nm were prepared by diluting concentrated protein solutions into 0.1 M sodium phosphate, 0.2 M NaCl buffer; 2-mercaptopoethanol was added to a concentration of 10^{-3} M.

Instrumentation

Fluorescence emission spectra were obtained with an SLM-8000 fluorometer (SLM Industries, Urbana, IL). The proteins were excited at 300 nm to isolate the tryptophan fluorescence. Fluorescence decay measurements were obtained by time-correlated single-photon counting. Detailed descriptions of the instrumentation and data collection are given elsewhere [12]. The frequency-doubled output of a Spectra Physics 3640 mode-locked Nd:YAG laser was used to pump synchronously a rhodamine 6G dye laser that was cavity dumped at 800 KHz. A Hamamatsu R1564U proximity-focused 12-μm 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-bandpass interference filters. Descriptions of the data analysis procedures have been given previously [4].

RESULTS

Figure 2 shows that replacement of glutamine-105 by alanine clearly results in a large increase in fluorescence intensity of tryptophan-138 when either alanine or threonine is present at position 146. The value of the quantum yield for these species with alanine at position 105 is very close to that for tryptophan in aqueous solution. A red shift of the emission spectra is observed with all substitutions, but especially with threonine at position 146. This indicates that the tryptophan at position 138 has a more polar environment as is provided by exposure to solvent [1–3,7,8].

The ratio of $Q/\langle\tau\rangle$ is (for a well-behaved chromophore) the radiative lifetime (Table I). The variation in

Table I. Fluorescence Parameters for Four T4 Lysozyme Variants

	Q^a	λ_{max}	$\langle \tau \rangle$	$Q/\langle \tau \rangle$
105Q	0.044	335	1.4	0.031
146T	0.028	346	1.5	0.018
105A	0.120	342	5.1^b	0.024
105A/146T	0.140	347	5.3	0.026

^{a}Based on Q=0.12 for tryptophan at pH 7.4.
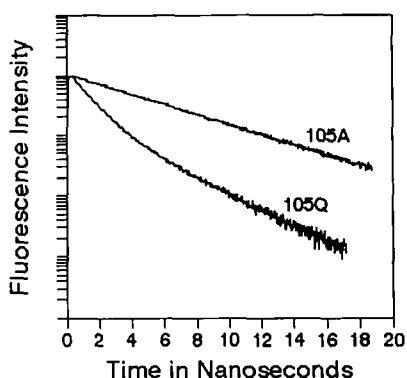^{b}Single exponential.

Fluorescence Decay of 105Q vs 105A

Fig. 3. The time dependence of the fluorescence of T4 lysozyme species with only tryptophan-138 and with alanine or glutamine at 105.

Table II. The Bimolecular Quenching Rate Constants for the Amino Acids for Quenching the Steady-State Fluorescence of Acetyl Tryptophan [13]

		k $(10^9 \ M^{-1} \ s^{-1})$
S–S	Disulfide	Large
H	Histidine	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
S	Serine	0.3
T	Threonine	0.2
G	Glycine	0.2
K	Lysine	0.2
V	Valine	0.2
A	Alanine	0.1

this ratio for the species given here (especially the low value for 146T) probably reflects the loss of an unresolved short lifetime component.

The decay of the fluorescence versus time of the single tryptophan-containing species is clearly nonex-

ponential [4]. This is shown graphically in Fig. 3, where the time dependence of the fluorescence of this species is compared with that observed for the protein in which glutamine-105 has been replaced by alanine. The decay of the alanine-105 species is essentially single exponential. The decrease in χ^2 upon analysis using two exponential components is less than 0.1.

DISCUSSION AND CONCLUSIONS

Amino acids are known quenchers of tryptophan fluorescence [13]. The bimolecular quenching rate constants span a range of almost 30 from the most effective to the least effective quenchers. These values are shown in Table II. Glutamine is a relatively effective quencher, while alanine is not. (It should be noted that the data in Table II refer to the free amino acids in their zwitterionic form; the values observed for alanine and valine may refer to the ammonium group.)

The increased quantum yield of fluorescence of tryptophan-138 in T4 lysozyme upon replacement of glutamine-105 (a moderate quencher) by alanine (a weak quencher) is consistent with the solution results in Table II. The value of $Q = 0.12$–0.14 suggests that there is little or no quenching in the alanine species.

To associate the change in fluorescence properties with the specific interaction of the tryptophan residue with the neighboring glutamine, it is necessary to show that there are no relevant structural differences in the mutant proteins. That this is the case is demonstrated by the X-ray diffraction structure for the glutamine-to-alanine mutant protein (containing all three tryptophan residues) [7].

The change in the form of the fluorescence decay of the wild-type single-tryptophan protein (105Q) from a very nonexponential form to a single-exponential decay is especially interesting. There has been considerable discussion over the years as to the origin of the nonexponential fluorescence decay behavior of proteins containing a single tryptophan. A recent editorial discussion [14] gives most of the key references, which are not repeated here.
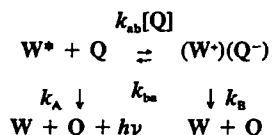
The most prevalent model as to the origin of the nonexponential behavior of protein fluorescence is that it derives from the coexistence of two (or more) conformational states of the protein, each with a distinct fluorescence lifetime [14]. If these hypothetical conformational states interconvert, it must be on a time scale that is long compared with the fluorescence lifetime for two decay components to be observed. According to this model the amplitudes of the decay components are re-

lated to the fraction of each species present in the sample.

For the case of T4 lysozyme containing only tryptophan-138 and glutamine at position 105, the amplitudes of a double-exponential fit are roughly 80 and 20% (τ = 1.1 and 3.1 ns). According to these values, this model requires that there be an appreciable population of two species with distinct tryptophan environments separated by a barrier of at least 3 kcal/mol and a free energy difference of about 0.8 kcal/mol. The high barrier precludes the possibility that the two species are slightly different indole ring orientations in the same local minimum.

Whatever the origin of this nonexponential decay behavior, we see from the results in Fig. 3 that this phenomenon is eliminated by replacement of the quenching glutamine residue by alanine.

An alternative model for the origin of this nonexponential decay behavior has recently been proposed [1]. This model derives from the fact that the mechanism by which quenching occurs is an electron transfer process [13]. The variability in bimolecular rate constants for amino acids (Table II) indicates that this process involves an equilibrium. This suggests the following kinetic scheme [1]:

$$\begin{array}{ccc} & k_{ab}[Q] & \\ W^* + Q & \rightleftarrows & (W^+)(Q^-) \\ k_A \downarrow & k_{ba} & \downarrow k_B \\ W + Q + h\nu & & W + Q \end{array}$$

The species involved in this kinetic scheme are tryptophan in its ground and excited states (W and W*), a quencher Q, and the contact radical ion pair (or CRIP), $(W^+)(Q^-)$. The key feature in this scheme that differs from those previously proposed is the reversibility of the quenching reaction. Emission resulting from back electron transfer is called recombination luminescence and is well-known [15,16]. Previous models involving dielectric relaxation or other excited-state processes differ in a kinetic sense from this scheme due to the lack of emission from the CRIP species, i.e., k_B is purely nonradiative. This kinetic scheme results in a double-exponential decay for the emission [17].

Reasonable values for the rate constants can be determined from the fluorescence amplitudes and decay rates [1]. For the particular case of the T4 lysozyme species 105Q, a double-exponential fit to the data yields amplitudes of 0.79 for a 1.1-ns component and 0.21 for a 3.1-ns component. By hypothesis, removal of the quenching glutamine residue should reduce the decay rate to k_A. From the crystal structure determination [7],

there is little if any change in the environment of tryptophan-138 for the glutamine-to-alanine substitution at 105. We conclude that 105A provides a good model for the unquenched case and thus that k_A = $1/\tau_{105A}$ = 1/5.1 $\approx$ 0.2 ns^{-1}. The analytic solution of this kinetic scheme [17] then yields values of k_B = 0.35 ns^{-1}, $k_{ab}[Q]$ = 0.6 ns^{-1}, and k_{ba} = 0.10 ns^{-1}. (It is not possible or necessary to separate $k_{ab}[Q]$ into its factors.) According to this analysis the equilibrium constant for the electron transfer process from excited tryptophan to glutamine is about 6.0. This ratio presumably varies considerably from one amino acid to another.

The main point of the present publication is that this explanation for the nonexponential fluorescence decay depends on the presence of the quenching group, Q. According to this model removal of the group Q will lead to a single-exponential decay with rate k_{AB}. The fact that this is what is observed lends support to this model but does not conclusively prove it.

This model naturally generalizes to more complicated (multiexponential) decay behavior for tryptophan environments in which more than one quencher group is present. Qualitatively, one would expect multiexponential behavior for those cases where the fluorescence is highly quenched due to interactions with multiple quenchers. The presence of other quenching residues in the vicinity of tryptophan-138 (e.g., methionines 103 and 106) may be the origin of the nonexponential decay of the A146T/Q105A mutant and of the need for three components to obtain a satisfactory fit for the 105Q species (χ^2 = 1.1 for three components vs 1.5 for two components).

A generalization of this model can also accommodate the situation of a dependence of the temporal parameters on the emission wavelength. This time dependence of the emission spectrum has been observed for T4 lysozyme single-tryptophan mutants [4] as well as for other proteins. This is expected if a component of the emission is due to "radiative recombination" [16]. This process involves electron return from a charge transfer state of the pair (WQ). This charge transfer state may be stabilized by vibrational relaxation or dipolar relaxation and thus this component of the emission occurs at a longer wavelength.

The kinetic model proposed here does not depend critically on the hypothesis that quenching is due to electron transfer. What is necessary is that the quenching interaction (whatever its nature) results in a short-lived intermediate which retains sufficient energy to excite the indole chromophore, so that an equilibrium is established and that the intermediate is itself not emissive. For example, this quenching process could be proton

transfer to produce a tautomeric state of the (WQ) pair. At least one case is known in which reverse transfer of a proton results in subsequent emission [18]. The kinetic scheme is the same as postulated above if the tautomeric form does not emit.

This class of "reversible kinetics" models provides a "homogeneous" description of the nonexponential decay of protein tryptophan fluorescence that does not involve multiple-ground state conformational states and does not imply any emission components with negative preexponential factors.

ACKNOWLEDGMENTS

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