FLUORESCENCE AND ENERGY TRANSFER OF TRYPTOPHANS IN *APLYSIA* MYOGLOBIN

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ABSTRACT The fluorescence decay of tryptophan residues in apo and met *Aplysia limacina* myoglobin and sperm whale myoglobin were measured in aqueous solution at 10°–15°C. In all species, multiexponential behavior was observed in which the individual components displayed unique frequency-dependent emission characteristics. The results suggest that the tryptophan fluorescence in all met samples are quenched by rapid Forster energy transfer to the heme as predicted from the crystal geometry. Fluorescence from the apo protein is similar to that in solutions of free tryptophans. In addition, the fluorescence properties of the reversible thermal denaturation of *Aplysia limacina* met myoglobin was investigated between 25° and 75°C.

INTRODUCTION

In recent years there has been considerable interest in the use of tryptophan as an environmental probe in proteins (1, 2). Although tryptophan fluorescence has been shown to be a useful tool in such investigations, interpretation of the results of these studies is made more difficult because of the yet unresolved questions regarding the multiexponential nature of the fluorescence decays of free tryptophans and those present in proteins.

Before the development of highly sensitive photon counting techniques, free tryptophan and its simple derivatives were generally reported to exhibit single exponential fluorescence decays. When the more sensitive techniques were applied to the same species, it was found that many simple tryptophan compounds fluoresced with multiexponential decay behavior (3–6). The multicomponent behavior observed in these compounds was originally attributed to emission from two different tryptophan transitions (^1L_a and ^1L_b) (3), and then later to conformationally selective quenching of the excited indole moiety by the carboxylate and amino functional groups on the side chain (7). Such quenching is thought to involve either proton transfer or electron transfer between the indole ring and the side groups (4–6, 8). The occurrence of two rather than the expected three components in the fluorescence decay in certain species was rationalized by assuming one of the rotamer configurations to be energetically unfavorable or that similar quenching mechanisms occur for two of the rotamers (4–6).

The interpretation of the time-resolved fluorescence spectra is different when the tryptophan emission results from residues in heme proteins. In such macromolecules tryptophan fluorescence is expected to be quenched via efficient Forster energy transfer with the heme group. The effects of there being various rotamer populations on the fluorescence decays, for example, is negligible when the energy transfer rate greatly exceeds the various fluorescence rates because the observed fluorescence rate is the sum of the intrinsic rate for the rotamer and the energy transfer rate. The hemes undergo rapid internal conversion to the ground state after the energy transfer process. The different tryptophans are expected to transfer energy at different rates because of their different distances and orientations with respect to the heme. For example, different ferrous and ferric derivatives of sperm whale myoglobins exhibit this multiexponential fluorescence decay behavior where the dominating lifetime components match those predicted by the Forster rate equation for each tryptophan (9). However, these same myoglobins also gave ~10% or less of long-lived fluorescence decay components with lifetimes on the order of several nanoseconds, the same magnitude as that observed in the apo proteins (9).

In a related study on human hemoglobin, Szabo et al. (10) used time-correlated single photon counting techniques to study tryptophan fluorescence decays. Their results indicated the presence of three decays with a short (~90 ps) component, which was attributed to energy transfer via Forster coupling to the heme, and two longer lived components (~1.8 and 5 ns), which must be essentially uninfluenced by energy transfer. In these experiments, the two long components comprised ~37 and 33% (human met hemoglobin) of the total integrated fluorescence intensity but <10% of the emission signal at zero time. The authors suggested that the long-lived fluorescence results from different rotamers, only one of which is capable of energy transfer to the heme within the lifetime of its excited state, due to orientational factors.

The question arises as to whether at ambient temperature a tryptophan could maintain in the protein a geometric relation to the heme such that energy transfer was prohibited. For this situation to prevail the tryptophan transition dipole would be required to be fixed perpendicular to the heme plane for periods of time comparable to the fluorescence lifetime of a few nanoseconds. Recent energy transfer calculations of Forster coupling (sperm whale myoglobin [sw Mb]) as a function of restricted rotation about the C(β)-C(γ) bond (9), as well as computer-simulated trajectories (11), have not found such configurations. It therefore appears that a purely orientational effect is inadequate to explain the long-lived (nanosecond) components of tryptophan fluorescence in heme proteins. For example, the trajectory calculations have so far failed to predict long-lived heme-tryptophan configurations for which the energy transfer rates match the aforementioned long-lived components observed experimentally. In addition, experimental evidence exists that seems to preclude the possibility of these long components resulting from some apo protein present in solution. Addition of excess hemin to solutions of sw myoglobin did not significantly reduce the fluorescence contribution from the long-lived components (9).

Here we present the results of time-correlated single photon counting experiments, which are used to study both frequency resolved and time domain fluorescence spectra of met and apo *Aplysia limacina* myoglobin (Mb). The choice of *Aplysia* Mb was suggested by the following considerations: (*a*) the two tryptophans, albeit fairly close

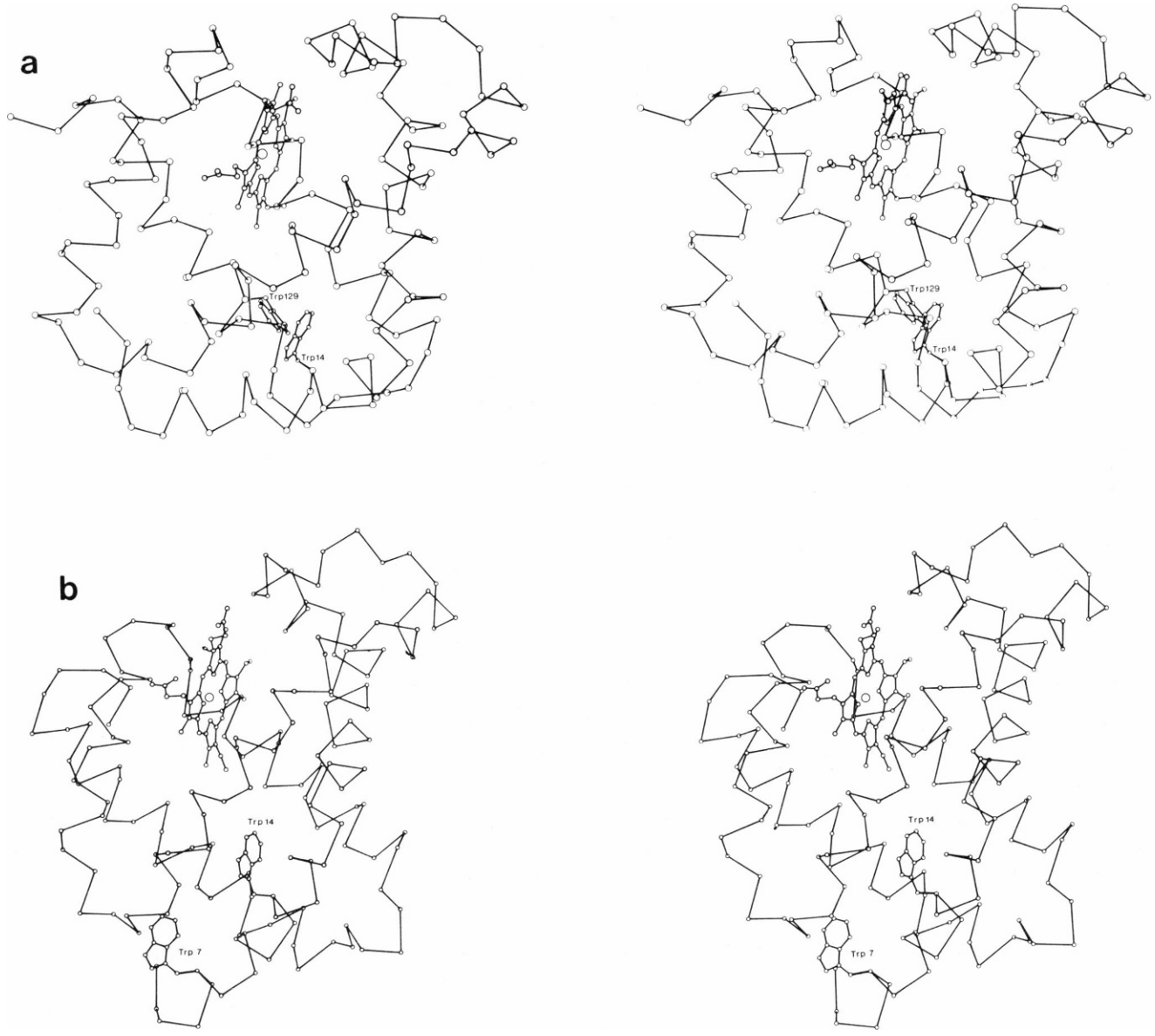

FIGURE 1 (*a*) C-Alpha stereo view of *Aplysia* Mb backbone. Both Trp(A)14 and Trp(H)129 are labeled. (*b*) C-Alpha stereo view of sperm whale Mb backbone. Both Trp(A)7 and Trp(A)14 are labeled.

in their three-dimensional structure, are on different helices Trp (A) 14 and Trp (H) 129 (12), whereas in sperm whale Mb they are both on the A helix (Fig. 1, *a* and *b*); and (*b*) this protein undergoes a fully reversible thermal denaturation with increase in the steady-state fluorescence emission upon warming (13). The effect of the thermal denaturation on the time-resolved fluorescence of *Aplysia* myoglobin is interesting since the two tryptophans reside on different chains. If the different fluorescence components each resulted from a particular tryptophan, one would expect the behavior of the individual components to be sensitive to the different tryptophan environment upon denaturation. As the protein unfolds upon warming, one expects that the change in the average distance from the heme will be different for the two tryptophans, enabling one to identify a particular fluorescence component with its tryptophan source. For example, we might guess that the fluorescence resulting from the tryptophan on the A helix be more sensitive to the thermal denaturation process since this chain resides on the protein surface and would be expected to unfold more readily.

We compare the results on this protein with fluorescence data obtained here and by others (9) for various derivatives of sperm whale Mb (Trp [A] 7 and Trp [A] 14), and we discuss our findings in terms of the likely modes of tryptophan fluorescence relaxation based on the x-ray diffraction structures (12, 14, 15).

EXPERIMENTAL

Aplysia met Mb was purified from the buccal muscle of the mollusc by ammonium sulfate fractionation (16) followed by preparative isoelectric focusing. Analytical and preparative isoelectric focusing of *Aplysia* met Mb were performed on a Multiphor LKB 2117 apparatus (Bromma, Sweden), applying a pH gradient of 4–6 (17), since the isoelectric point of this protein is 4.75 (18). Coomassie Blue was used as a stain for the protein; the homogeneity of the heme-carrying component was checked by direct observation of the visible absorption. Crystalline sperm whale met Mb from Sigma Chemical Co. (St. Louis, MO) was purified using the method of Hochstrasser and Negus (9). Both *Aplysia* met Mb and sperm whale met Mb were passed through a Sephadex G25 column, followed by exhaustive dialysis against distilled water.

The purity of the protein samples is critical for accurate determination of the fluorescence lifetimes. In fact, samples of *Aplysia* met myoglobin that were not purified by isoelectric focusing were heavily contaminated (up to 30%) by kinetic components whose lifetimes were 10–20 times longer than the short-lived component, which is the dominant species in the purified samples.

The apo derivatives of both myoglobins were prepared from the purified heme-containing proteins by acid splitting and extraction with 3-pentanone and/or acetone at −20°C as previously reported (19, 20). The azide derivatives of *Aplysia* and sperm whale Mb were prepared by adding sodium azide to a final concentration of 0.2 M, which is sufficient for the complete formation of the azide adduct of both the heme proteins (21, 22). Protein samples were buffered to pH values ranging between 6.8 and 10, using either 0.025 M Tris or sodium tetraborate, and adjusted in concentration to give optical densities of 0.5–0.9 through a 1-mm pathlength cell at 280 nm.

The photon counting system used in these experiments consists of a frequency-doubled, cavity-dumped dye laser synchronously pumped by a mode-locked argon ion laser. The cooled samples (10°–15°C) were excited by the doubled output of the dye laser, and front face fluorescence (magic angle) was collected, filtered through a cutoff filter (model WG-305; Schott American Glass & Scientific Products, Inc., Yonkers, NY) and dispersed with a 275–0.2 meter holographic monochromator (McPherson Instrument Co., Acton, MA) with a 6-nm bandpass. The output of the monochromator was coupled to a microchannel plate detector (Hamamatsu Corp., Middlesex, NJ). With this arrangement, instrument functions (determined by detection of resonant scatter from the samples) of 120 ps for R6G and 180 ps for R560 were typical. Multiexponential decays of the form $F(t) = \Sigma A_i \exp - (t/T_i)$, where $F(t)$ is the fluorescence intensity at time t, A_i the preexponential factor, and T_i the lifetime of the ith component, were convoluted with the instrument function and iteratively fit to the experimental curves. Fits with a correlation coefficient of 1.2 or better were considered acceptable. Three exponential components were included in our fitting routines.

RESULTS

Fluorescence Decay

In both the apo and met preparations of sperm whale and *Aplysia* Mb the principal contribution to the fluorescence results from the tryptophans. In the former case tyrosine emission is discriminated against by the use of interference filters and selective monitoring of fluorescence emission wavelength. *Aplysia* Mb lacks tyrosine residues (23) and no added precautions were necessary.

Aplysia Apo Mb. The decay of the fluorescence intensity was found to be best described as a sum of three exponentials whose relative weights and lifetimes showed a marked dependence on detection wavelength. Table I lists the best fit parameters for various fluorescence detection wavelengths tuned across the emission band (310–390 nm) with 283-nm excitation. One notes that the short (subnanosecond) emission is shifted to shorter wavelengths and the intermediate (~1 ns) component is shifted to longer wavelengths relative to the long-lived component, whose relative weight is approximately constant across the emission spectrum. In addition, one notes the marked lengthening of the lifetimes of the two long-lived (nanosecond) components with increasing detection wavelength, while the best fit

TABLE I
TIME-RESOLVED FLUORESCENCE OF TRYPTOPHAN(S) IN APO *APLYSIA* Mb AT 283-NM EXCITATION AS A FUNCTION OF EMISSION WAVELENGTH BASED ON A TRIEXPONENTIAL FIT

	Emission wavelength (nm)								
	310	320	330	340	350	360	370	380	390
A_1	0.305	0.216	0.177	0.160	0.142	0.142	0.131	0.119	0.112
A_2	0.394	0.516	0.511	0.568	0.572	0.539	0.524	0.582	0.560
A_3	0.301	0.268	0.310	0.273	0.280	0.317	0.344	0.298	0.335
T_1	0.135	0.201	0.238	0.262	0.257	0.204	0.203	0.342	0.191
T_2	1.16	1.44	1.54	1.63	1.65	1.61	1.60	2.83	1.75
T_3	3.16	3.99	3.86	4.53	4.66	4.58	4.59	5.19	5.09

The preexponential factors are normalized to one at each emission frequency. Estimated error for both preexponential factors and lifetimes (given in nanoseconds) is <8% for all data presented here.

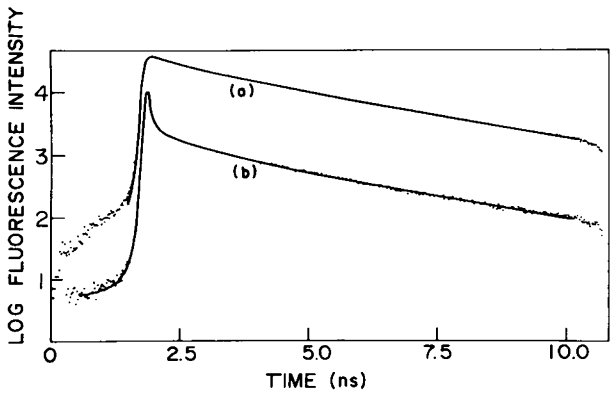

FIGURE 2 (*a*) Log plot of the fluorescence emission at 335 nm from apo *Aplysia* myoglobin with 283-nm excitation. Time scale is 10 ns full scale. The *solid line* indicates the calculated fit to the experimental points. (*b*) Same as *a* for 335-nm emission from *Aplysia* met Mb with 283-nm excitation.

parameters for the short-lived component show no such trend. Fig. 2 *a* shows a typical fluorescence decay for apo *Aplysia* Mb at 335 nm with 283-nm excitation.

The fluorescence anisotropy of *Aplysia* apo Mb was observed to decay with a lifetime of 4.5 ± 0.25 ns with an initial extrapolated anisotropy $r(0)$ of 0.09, which is somewhat smaller in magnitude than reported by Hochstrasser and Negus for solutions of apo sperm whale Mb at 272-nm excitation (9).

Aplysia Met Mb. In Fig. 2 *b* a typical fluorescence decay from *Aplysia* met Mb is shown, which is markedly different from that observed for the apo *Aplysia* preparation. Again, our best fit is obtained when we model the decay as a sum of three exponentials, with a short-lived component decaying with a lifetime of 15–30 ps, which comprises ~95% of the total initial fluorescence at 335 nm, and two longer lived components, which together make up the other 5% of the initial fluorescence and decay on the nanosecond time scale. The two longer components decay with lifetimes comparable to those observed here in *Aplysia* apo Mb samples and those for aqueous tryptophan solutions (4–6). Table II shows the best fit parameters for

TABLE II
EXCITATION SPECTRA OF TRYPTOPHAN EMISSION (AT 335 NM) IN MET *APLYSIA* Mb. A_is ARE THE NORMALIZED PREEXPONENTIAL FACTORS AT A GIVEN EXCITATION WAVELENGTH (15°C)

Excitation	Excitation wavelength (nm)		
	275	283	292
A_1	0.960	0.960	0.960
A_2	0.023	0.020	0.016
A_3	0.016	0.016	0.024
T_1 (*ns*)	0.022	0.016	0.028
T_2	0.823	0.820	0.790
T_3	3.34	3.01	2.90

TABLE III
EXCITATION SPECTRUM OF TRYPTOPHAN EMISSION (AT 335 NM) IN APO SPERM WHALE MYOGLOBIN

	Excitation wavelength (nm)		
	275	286	297
A_1	0.230	0.243	0.335
A_2	0.423	0.419	0.352
A_3	0.346	0.337	0.312
T_1 (*ns*)	0.288	0.279	0.207
T_2	1.30	1.33	1.35
T_3	3.62	3.76	3.90

the fluorescence decay at 335 nm at three different excitation wavelengths. The slightly higher values of the preexponential factors for the long-lived components at 292-nm excitation suggest that these components may result from tryptophans in an environment more polar than the short-lived component.

Sperm Whale Apo Mb. Table III shows the fluorescence decay parameters obtained for best fits at different excitation frequencies on sperm whale apo Mb. We observe the decay as a sum of three similarly weighted exponentials with two nanosecond components and one subnanosecond component, the lifetimes of which are quite similar to the corresponding lifetimes in *Aplysia* apo Mb. No remarkable excitation wavelength dependence on either the lifetimes or preexponential factors is noted. To the best of our knowledge, only biexponential decays have been previously reported for apo sperm whale Mb (9).

Sperm Whale Met Mb. Table IV shows the decay parameters of the three component fluorescence observed at 335 nm for sperm whale met Mb with 272-nm excitation. The short-lived (100 ps) component is actually known from streak camera results to consist of two fast components (16 and 135 ps), which result from Forster energy transfer from the two tryptophans to the heme (9). The long-lived part of the decay is composed of two components of ~0.8 and ~3 ns, and must result from

TABLE IV
BEST FIT PARAMETERS FOR THE MULTIEXPONENTIAL EMISSION AT 335 NM FOR DERIVATIVES OF SPERM WHALE AND *APLYSIA* MET Mb

Protein	Sw met Mb	Sw met Mb (azide)	*Aplysia* met Mb (azide)
Excitation	272 nm	283	283
A_1	0.958	0.853	0.975
A_2	0.030	0.138	0.015
A_3	0.012	0.005	0.010
T_1 (*ns*)	0.111	0.096	0.019
T_2	0.872	0.222	0.610
T_3	3.08	2.83	3.19

tryptophans that are somehow prohibited from transferring a significant fraction of their excitation to the heme.

Azide Derivatives of Sperm Whale and Aplysia Met Mb. Table IV shows the best fit parameters for the 335-nm fluorescence emission from the azide derivatives of both sperm whale met Mb and *Aplysia* met Mb, for the sake of comparison. In both cases the bulk of the fluorescence results from components decaying with subnanosecond lifetimes, while each shows trace emission from a ~3-ns component. The lifetime values of the intermediate-lived components are considerably different from those observed for the corresponding unliganded species under similar conditions.

Thermal Denaturation of Aplysia Met Mb. In the denaturation experiment of *Aplysia* met Mb, protein samples were buffered at pH 8.6 with 0.025 M Tris-HCl, and 6% (vol/vol) of *n*-butanol was added (13). The fluorescence decay of tryptophan residues (335-nm emission with 283-nm excitation) was measured as a function of temperature between 25° and 70°C. The results in Table V show a long-lived (~3-ns) component that is insensitive to the denaturation process and an intermediate-lifetime component whose relative weight increases monotonically with temperature until denaturation is achieved, when the increase is more abrupt and is accompanied by a decrease in the lifetime. The short-lived decay exhibits a temperature behavior complementary to the intermediate component, its lifetime increasing from 22 to 134 ps as the temperature was raised from 25° to 68°C. The reversibility of the denaturation is illustrated by the return to the initial (low temperature) parameters upon cooling of the sample, as shown in the last column of Table V. The total number of fluorescence counts dropped by a factor of about two between 25° and 56°C. Over this range there is a color change and also some precipitation of the protein, but we have not quantified this behavior.

TABLE V
TEMPERATURE DEPENDENCE OF THE FLUORESCENCE DECAY PARAMETERS OF *APLYSIA* MET Mb IN AQUEOUS-BUFFERED SOLUTIONS (TRIS 8.6) WITH 6% (VOL/VOL) OF *n*-BUTANOL ADDED

	Temperature (°C)					
	25	33	44	56	68	15
A_1	0.881	0.864	0.823	0.624	0.627	0.915
A_2	0.067	0.079	0.113	0.330	0.311	0.054
A_3	0.051	0.057	0.064	0.051	0.062	0.030
T_1 (*ns*)	0.020	0.024	0.040	0.113	0.134	0.066
T_2	0.863	0.862	0.804	0.574	0.595	0.743
T_3	2.78	2.77	3.16	2.64	2.37	2.89

The last column corresponds to the protein that has been denatured and then cooled to 15°C.

DISCUSSION

The results obtained here for the fluorescence decay of tryptophan in apo *Aplysia* Mb are consistent with the rotamer models used to explain the multiexponential fluorescence decays observed in aqueous solutions of tryptophan and its derivatives (4–8). In this model, the excited indole nucleus may react preferentially with a side chain moiety, depending on its rotational configuration, to yield distinct spectra with different fluorescence lifetimes for the various rotational species. In apo *Aplysia* Mb, we observe three spectrally distinct species with a subnanosecond (~200 ps) fluorescence component that is shifted to shorter wavelengths with respect to the two longer lived (~1.5 and 4 ns) components. In all three species, the lifetimes are observed to be dependent on detection wavelength. There is a marked tendency toward longer lifetimes at longer wavelengths on the fluorescence band.

Fluorescence of tryptophan residues in heme proteins is expected to differ from that observed in the apo protein in that the lifetimes directly reflect the tryptophan-heme energy transfer rate. In view of the heme tryptophan distances in various myoglobins (~10–20 Å) and hemoglobins, one expects singlet energy transfer to be dominated by Forster resonance coupling (24). The data obtained here for met *Aplysia* myoglobin, as well as those obtained previously by others (9) for sperm whale myoglobin, seem to indicate that the bulk of the excited tryptophan population participates in subnanosecond energy transfer with the heme group. The rate constant for energy exchange between two species coupled by the Forster transfer mechanism is given by:

$$k_i = \sum_h \frac{8.8 \times 10^{-5}\,\kappa_{ih}^2}{r_i^6 n^4 \tau_{ri}} \int F_i(\lambda)\epsilon_h(\lambda)\lambda^4 d\lambda, \qquad (1)$$

where κ^2 is the usual dipole–dipole orientation factor, n is the index of refraction of the media, τ the radiative lifetime of the donor species, and r the distance between the donor and the acceptor. The Forster integral at the right of Eq. 1 has the value of 3.58×10^{14} quanta·nm^6·mol^{-1}, obtained from the known heme molar decadic extinction coefficient (ϵ) at wavelength (λ) and the fluorescence data of Anderson et al. (25). Assuming an estimated value of 20 ns for the radiative lifetime of tryptophan and using the known crystallographic parameters (12) for r and κ, we calculate transfer times of 8.8 and 11.4 ps for Trp (A) 14 and Trp (H) 129, respectively. In addition, to account for different orientations of the tryptophan transition moment dipole with respect to the heme, we calculated the transfer rate as a function of the angle of rotation (2π) about the Trp C(β)–C(γ) bond, and found no region where transfer times were not predicted to be subnanosecond. The results of similar calculations have been previously reported for sperm whale Mb (9) for which similar transfer times were obtained. In addition, energy transfer rates have been calculated for sperm whale myoglobin based on computer-simulated trajectories (11). In the trajectory calculations,

no stable configuration was yet found that could account for nanosecond energy transfer times in sperm whale Mb.

The proximity of the two tryptophans in *Aplysia* Mb (Fig. 1 *a*) suggests the possibility that trp-trp energy transfer might influence the decay characteristics. Using the fluorescence and absorption data of Anderson et al. (25) and the crystallographic data of Bolognesi (12) we find a maximum overlap integral of 6.45×10^{12} quanta·nm^6·mol^{-1} at 300 K and the separation between tryptophan centers of 6.6 Å lead to a transfer time of 23.5 ns when the crystallographic parameters are used in the calculation. However, when we calculate the transfer time as a function of the angle between the two dipoles we find that the transfer times are <4 ns when the angle is increased by 15° or decreased by 8° from the equilibrium (crystallographic) angle of 54.1°. The calculated minimum transfer time was 23 ps. While the longer value of the transfer time is beyond the time scale of our experiment, the more rapid transfer times would be observable in a fluorescence polarization experiment. Trp-trp energy transfer would be expected to alter the decay of the fluorescence polarization in both the apo and met Mb samples. The values of the anisotropy extrapolated to time zero for met sperm whale Mb were previously reported as 0.16- for 265 nm-excitation (9), 0.22 for 287-nm excitation (26), and in the case of apo sperm whale Mb a value of 0.15 was reported for 272-nm excitation (9). The value of 0.09 observed in the present work is significantly lower than the previously measured values of $r(0)$ for heme proteins. One possible reason for this discrepancy is that trp-trp energy transfer occurs rapidly compared with the fluorescence lifetime of the apo Mb. If θ is the angle between the transition moment dipoles of the two trps then the transfer anisotropy $r_{1\to2}$ is given by (27)

$$r_{1\to2} = 1/5(3\cos^2\theta - 1). \quad (2)$$

In the absence of other processes that give rise to a decay in the anisotropy, $r(t)$ following a delta function excitation pulse, will be given by (28)

$$r(t) = 1/2[r(0) - r_{1\to2}]\exp^{-2kt} + 1/2[r(0) + r_{1\to2}], \quad (3)$$

where k is the excitation energy transfer rate constant. Using the value of 54.1° for θ, and a value of 0.15 for $r(0)$ we find that $r(t)$ would decay to a value of 0.088 after one transfer lifetime. We would therefore expect to observe this component superimposed on the overall anisotropy decay resulting from protein motion. With the present sensitivity of our photon counting apparatus we would expect to be able to resolve decays in the anisotropy of at least 90 ps. Also, any component of <90 ps would be expected to produce an abnormality in the weighted residuals and effect chi squared for our fit. Our results indicate that the data are best fit by a single exponential with a lifetime of 4.5 ns and there is no indication for the existence of any shorter component. As this lifetime is on the order of the rotational correlation time for the protein it would appear that energy transfer between the two trps does not occur within the fluorescence lifetime of the protein. Since most orientations of the transition moment dipoles give rise to subnanosecond energy transfer, these results suggest that the tryptophans are restricted to be near their crystalline orientations. We cannot yet exclude that the energy transfer occurs on a time scale too rapid for us to observe.

The short-lived (15–30 ps) component we observe in *Aplysia* met myoglobin, which is absent in the apo preparation, is directly attributable to Forster energy transfer to the heme. Ideally one would expect to observe two short-lived components reflecting the different transfer rates from each of the two tryptophans Trp (A) 14 at r = 14 Å, Trp (H) 129 at r = 16 Å from the heme (see Fig. 1). The similarity of the two rates allows us to observe only one average component with the time resolution of our photon counting system. In addition, the discrepancy between the calculated and observed transfer times can likely be accounted for by the fact that crystalline parameters were used and that fixed rather than motionally averaged tryptophan rotational conformations were assumed. The presence of 1–3% of long-lived (~3 ns) emission from these samples seems to indicate that a small fraction of the generated tryptophan population is not transferring excitation energy to the heme. As evidenced by calculated transfer times, such long-lived behavior is inconsistent with the certainty of Forster coupling in these proteins. This suggests that either some of the fluorescence results from tryptophans much farther from the heme than indicated by the crystal structure, or that some other process occurs that inhibits energy transfer and yet still gives rise to the characteristic tryptophan fluorescence. This fluorescence apparently does not arise from free apo protein in equilibrium with the heme-containing protein since in both the sperm whale Mb work (9) and the *Aplysia* Mb results presented here, addition of an excess of hemin to the sample did not significantly alter the fluorescence lifetime components. It therefore appears that some other mechanism is limiting the energy transfer, although it cannot be excluded that a small population of molecules with incorrectly bound heme may exist.

In addition to the long-lived (~3 ns) decay, we observe a ~0.8-ns component that accounts for another 1–3% of the total initial fluorescence. Again this component is not predicted by energy transfer calculations that consider only the crystalline parameters and therefore would require the persistent existence of protein configurations in which the interactions between the tryptophan and heme transition moment dipoles differ substantially from that predicted by the crystal structure. What is significant about these long-lived components is that while they comprise only a small fraction of the total initial time-resolved fluorescence, together they would account for ~80% of the emission in a steady-state experiment (63%

for the ~3-ns component and 17% for the ~0.8-ns component).

Increasing the distance between the tryptophan and heme by a factor of two would decrease the rate of energy transfer by 64, which is sufficient to increase the fluorescence lifetime to ~1 ns. The ambiguity of the effects seen here and with myoglobin (9) and hemoglobin (10) earlier are explained if the protein undergoes large amplitude conformational changes to an open structure in which the A-helix is unfolded and its associated tryptophans are more distant from the heme. To study this effect, we studied the fluorescence properties of *Aplysia* met Mb at various stages of thermal denaturation of the protein. Initially we observe a lower value of the preexponential for the ~20-ps component at 25°C in the 6% butanol solution as compared with the (Tris) aqueous-buffered protein samples, indicating that some degree of denaturation at room temperature occurs in the mixed solvent. The gradual drop in this preexponential factor with increase in temperature, together with the increase in lifetime at temperatures below the critical denaturation temperature, probably reflects the increased thermal motion of the protein chains. The more abrupt change that we observe at the critical denaturation temperature appears to reflect the decrease in energy transfer rate with the increase in Trp-heme distance as the protein chains unfold. The behavior of the intermediate (~0.8 ns) component appears to be complementary to that of the short component. The increase in the weight of this component with temperature suggests that this fluorescence may indeed result from a protein structure distinct from that favored in the low temperature (undenatured) samples, although it is unclear why the lifetime decreases as the protein denatures.

In contrast to the subnanosecond components, the parameters for the long (~3 ns) decay indicate that the fluorescence from this component (which accounts for 1–3% of the total initial emission) is insensitive to thermal denaturation of the protein. This fact, coupled with the red shift that we observe for this component in the excitation spectrum of *Aplysia* met Mb (Table II), suggests emission from tryptophans in a more aqueous environment. The possibility that this emission results from a less favorable long-lived conformer is not supported by the molecular dynamics or energy transfer calculations. While we have observed evidence of such conformers in the apo protein, and would expect that they exist in the corresponding heme protein, their presence does not explain the long-lived fluorescence decay. Because the heme transition is doubly degenerate, with the two transition moment dipoles arbitrarily located in the heme plane at right angles to one another, there is a very narrow range of tryptophan orientations that do not lead to efficient (subnanosecond) energy transfer. In addition, in the apo protein the relative weights of the short, intermediate, and long-lived components immediately after excitation are roughly 18, 51, and 31% at 330 nm. The insertion of the heme is unlikely to result in such a drastic reapportionment of the relative rotamer populations such as to yield a rotamer with only 3% of the total population. This suggests that the 3-ns fluorescence component results from some other source. The presence of free normal apo protein would have been detected with the addition of excess hemin, so our results exclude this as the origin of the long-lived fluorescence. Since our results were obtained on a system that is homogeneous in isoelectric focusing, we suspect that the impurities may constitute a considerable fraction of the fluorescence observed in a steady-state experiment. In view of the similarities in structure between *Aplysia* and sperm whale Mb, it is quite likely that the long lifetime fluorescence component(s) observed in the latter protein (9) are also the result of impurities that are not removed by standard purification techniques.

Free tryptophan is known to undergo several well documented photochemical reactions including photoionization, charge transfer, and proton transfer to the side chain moieties, all of which have been suggested as possible mechanisms for the multiexponential fluorescence decays observed in these species (4–6, 8). It is possible that similar mechanisms may take place in the protein environment, which is rich in other amino acids that are known to quench tryptophan fluorescence (e.g., His, Lys, Gly). The tryptophan might form a radical cation that would inhibit energy transfer and on recombination, could give rise to the long-lived decays observed in these proteins. However, since these photochemical reactions involve interactions between specific moieties and the indole nucleus one could hardly expect such photoproduct configurations to be insensitive to the drastic structural changes that occur on denaturation of the protein.

CONCLUSIONS

Tryptophan fluorescence from *Aplysia* met Mb decays with a multiexponential behavior similar to that observed in other heme proteins. The short (15–30 ps) component, which comprises ~95% of the initial fluorescence intensity and which is absent in the apo protein, is attributed to relaxation via Forster energy transfer with the heme. The intermediate component, which decays on a subnanosecond (~0.8 ns) time scale, would not be expected as a result of Forster coupling if the protein adopted its structure determined by x-ray diffraction. However, this component could result from tryptophan species with orientations other than those suggested from the crystal geometry, and does appear to be affected by changes in the protein structure coupled to thermal denaturation. The long-lived component, which decays with a lifetime of ~3 ns and accounts for nearly 70% of the steady-state fluorescence, is insensitive to the thermal denaturation of the protein and appears to originate from tryptophans in an environment more polar than the short-lived components. We conclude that this emission results from impurities that, however, have not been completely removed by standard techniques.

In apo *Aplysia* Mb the fluorescence decay is again multiexponential, the lifetimes and spectral properties being consistent with the rotamer model proposed for fluorescence decays observed in free tryptophan. In addition, comparison of the fluorescence anisotropy decay obtained here for apo *Aplysia* Mb with those obtained by others (9) for sperm whale apo Mb suggests that energy transfer between the two tryptophans is not a factor in these experiments despite their proximity.

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