Fluorescence Lifetime Quenching Studies on the Accessibilities of Actin Sulfhydryl Sites†

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ABSTRACT: The fluorescence decay of *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine(1,5-IAEDANS) labeled G-actin exhibited two decay components, a major component of lifetime 17.3 ns that accounted for 96% of the fluorescence, and a minor component of lifetime 33.3 ns. Lifetime quenching studies by the addition of acrylamide yielded quenching rate constants, k_q, of 2.31×10^8 M^{-1} s^{-1} and 6.5×10^7 M^{-1} s^{-1} for the major and minor components, respectively. Upon polymerization of the labeled actin, only one lifetime of 19.3 ns was detectable. The presence of a minor component was revealed when acrylamide was added. The values of k_q for labeled F-actin decreased from those of G-actin to 1.35×10^8 M^{-1} s^{-1} and 1.52×10^7 M^{-1} s^{-1} for the major and minor components, respectively. Pretreatment of the G-actin with *N*-ethylmaleimide (which is known to react primarily with cysteine-373 in actin) substantially blocks the subsequent incorporation of 1,5-IAEDANS. These data were interpreted as follows: The major decay component for labeled G-actin is associated with a major labeling site, probably located at cysteine-373. The minor decay component corresponds to a minor labeling site (or class of sites) of unknown location. The major labeling site is more polar and more accessible than the minor labeling site. Upon polymerization, both sites become less accessible. Intensity quenching studies on labeled F-actin in the presence and absence of tropomyosin revealed that tropomyosin further decreased the accessibility of the major labeling site (k_q decreased by about 30%). This finding can be interpreted to indicate that tropomyosin binds to actin at a site near cysteine-373.

Studies on the quenching of protein fluorescence by externally added solutes such as iodide ions were first described by Lehrer (1967, 1971) and by Burstein (1968). Later, Weber and co-workers used molecular oxygen as a quencher for the intrinsic and extrinsic fluorescence of macromolecules (Vaughan & Weber, 1970; Lakowicz & Weber, 1973a,b). More recently, Eftink & Ghiron (1975, 1976a,b, 1977) carried out extensive studies on the quenching of protein fluorescence by acrylamide. Applications of the technique to the study of macromolecular conformations have been reviewed by Lehrer & Leavis (1978). The method enjoys a number of attractive features: (1) Like all fluorescence methods, only micromolar amounts of material are required. (2) It yields a factor that serves as a *quantitative* measure for the accessibility of a fluorophore. Thus, the degree to which a fluorophore is exposed to quenchers in the medium can be discussed in quantitative terms. (3) The measurements are very precise, such that even small changes in accessibility can be detected.

Heretofore, however, most fluorescence quenching studies were carried out on the intrinsic fluorescence of proteins (fluorescence from the tryptophan and/or tyrosine residues of proteins) by using steady-state intensity measurements. With the exception of a few cases, the interpretation of the data in such studies is not always straightforward. This is due to two reasons. (1) Most proteins contain several tryptophan and/or tyrosine residues. The data reflect a composite of the steric environments of each emitter. Although methods exist to deal with this situation (Lehrer & Leavis, 1978), it is difficult to resolve the accessibilities of each individual emitter. (2) Two processes contribute to the quenching of fluorescence intensity: a collisional process and a static process. Again, unless the protein contains a single emitter, it is usually difficult to resolve the two processes and obtain an unambiguous measurement for the accessibility by using intensity quenching measurements alone.

In this paper, we report fluorescence lifetime quenching *and* intensity quenching studies on 1,5-IAEDANS[1] -labeled actin, using acrylamide as the quencher. Actin is the major protein component found in the thin filaments of muscle. It is known to interact with myosin heads of the thick filaments, and with the regulatory proteins troponin and tropomyosin (see a review by Mannherz & Goody, 1976). Studies on the properties of actin itself and on its interactions with other muscle proteins will contribute toward understanding the mechanism of muscle contraction and its regulation. The choice of 1,5-IAEDANS (Hudson & Weber, 1973) as the extrinsic probe was governed by its selectivity for sulfhydryl groups, its relatively long lifetime, and its spectral properties which are such that its emission is well separated from the protein emission. We chose acrylamide (Eftink & Ghiron, 1976a) as the quencher because it is uncharged, it is a highly efficient quencher, and it does not denature proteins even when present in molar quantities. We used the lifetime quenching studies to resolve the presence of multiple decay components. By combining the lifetime quenching results with intensity quenching results, we were able to resolve the contribution of the static process from that of the collisional process (Lakowicz & Weber, 1973a; Eftink & Ghiron, 1976a,b).

Our results show that the fluorescence decay of AEDANS–G-actin contains two components. These components can be identified with a major labeling site located at cysteine-373, and a minor labeling site (or class of sites). The lifetime studies indicate that the major labeling site is more polar than the minor labeling site. The quenching studies indicate that the major labeling site is more exposed than the

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[1] Abbreviations used: 1,5-IAEDANS, *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; AEDANS-G-actin and AEDANS-F-actin, 1,5-IAEDANS-labeled G- and F-actin, respectively; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EDTA, sodium ethylenediaminetetraacetate; Ans, 8-anilino-1-naphthalenesulfonate; MalNEt, *N*-ethylmaleimide; AEDANS–MalNEt–G-actin, G-actin pretreated with MalNEt, followed by 1,5-IAEDANS labeling.

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minor one. Upon polymerization to form AEDANS–F-actin, both sites become less exposed. In the presence of tropomyosin, the major labeling site of AEDANS–F-actin becomes even more shielded from the medium, suggesting that tropomyosin binds to G-actin near that site. A preliminary account of this work was presented (Tao & Cho, 1978).

Theory. Owing to the "collisional quenching" process, the fluorescence lifetime of an emitter decreases in the presence of quenchers according to the Stern–Volmer equation (Stern & Volmer, 1919)

$$1/\tau = 1/\tau_0 + k_q[Q] \quad (1)$$

where τ_0 is the singlet lifetime in the absence of quenchers; τ is the lifetime at some quencher concentration [Q]; and k_q is the bimolecular Stern–Volmer quenching rate constant, which can serve as a quantitative measure for the accessibility of the emitter; k_q approaches zero at low accessibilities and approaches the diffusion-control upper limit at high accessibilities.

The fluorescence intensity of a single emitter is decreased by both the "collisional quenching" process and the "static quenching" process according to (see Lehrer & Leavis, 1978)

$$F_0/F = (1 + K_{sv}[Q])e^{V[Q]} \quad (2)$$

where F_0 and F are fluorescence intensities in the absence and presence of quenchers, respectively; $K_{sv} = \tau_0 k_q$ is the Stern–Volmer quenching constant; and V is the static quenching parameter that can be interpreted as the volume of a sphere surrounding the emitter within which the emission is quenched by the quencher immediately after excitation (see a discussion by Eftink & Ghiron, 1976b). Note that the initial slope (IS) of a F_0/F vs. [Q] plot is given by

$$\left[\frac{d(F_0/F)}{d[Q]}\right]_{[Q]=0} = K_{sv} + V \quad (3)$$

When multiple emission components are present, the equations for intensity quenching and the initial slope become (Lehrer & Leavis, 1978)

$$F_0/F = \left[\sum_{i=1}^{n} \frac{f_i}{(1 + K_i[Q]e^{V_i[Q]}}\right]^{-1} \quad (4)$$

and

$$IS = \sum_{i=1}^{n} f_i(K_i + V_i) \quad (5)$$

where f_i is the fractional contribution from each emission component to the total emission, n is the total number of components, K_i is K_{sv} for each component, and V_i is the V parameter for each component.

It might be remarked here that, since only collisional quenching contributes to lifetime measurements, a plot of $1/\tau$ vs. [Q] should be linear as long as the measured lifetimes are truly representative of an individual decay component. For intensity quenching measurements, the presence of static quenching causes the F_0/F vs. [Q] plot to curve upward (concave) from linearity, while the presence of multiple components causes the plot to curve downward (convex) from linearity.

Experimental Section

Materials. G-actin was prepared from rabbit skeletal acetone powder according to Spudich & Watt (1971). Tropomyosin was prepared from rabbit skeletal ether powder by the Bailey method according to Greaser & Gergely (1971). 1,5-IAEDANS was from Aldrich. Acrylamide (ultra-high purity electrophoretic grade) was purchased from Bio-Rad, Ans was from Eastman Kodak, and myoglobin, Hepes, ATP, EDTA, and MalNEt were from Sigma. The Ans–apomyoglobin complex was prepared according to Stryer (1965).

Methods. G-actin was labeled with 1,5-IAEDANS by incubating the protein (2 mg/mL) with a tenfold molar excess of the label at 4 °C for 12 h in a solution containing 2 mM Hepes at pH 7.5, 0.2 mM $CaCl_2$, and 0.2 mM ATP. The excess reagent was removed by gel filtration through a Sephadex G-25 column. The concentration of the AEDANS moiety was determined from the absorbance at 337 nm, by using an extinction coefficient of 6000 M^{-1} cm^{-1} (Hudson & Weber, 1973). Actin concentration was determined from the absorbance at 290 nm by using $A_{290}^{1mg/mL} = 0.63$ $(mg/mL)^{-1}$ cm^{-1} (Lehrer & Kerwar, 1972), and $M_r = 42300$ (Elzinga et al., 1973). For labeled actin, the absorbance of the AEDANS moiety at 290 nm must be subtracted from the total absorbance in order to obtain the true actin absorbance. It was estimated that $\epsilon_{290} = 0.21\epsilon_{337}$ from the published absorption spectrum of 1,5-IAEDANS-labeled β-mercaptoethanol (Hudson & Weber, 1973). Labeled F-actin was obtained by polymerizing labeled G-actin in 50 mM KCl and 2 mM $MgCl_2$. This polymerization was accompanied by an increase in the fluorescence of the AEDANS moiety. AEDANS–F-actin so prepared has the same viscosity and the same tryptophan emission spectrum as unlabeled F-actin.

Incorporation of MalNEt into G-actin was carried out according to Elzinga & Collins (1975). The amount of MalNEt incorporated was estimated from the decrease in absorbance at 310 nm, by using $\Delta\epsilon_{310} = 580$ M^{-1} cm^{-1} (Lehrer et al., 1972). After removal of excess MalNEt by dialysis, the MalNEt–G-actin was subjected to 1,5-IAEDANS labeling following the procedure described earlier. A G-actin control was subjected to exactly the same series of treatments, except that MalNEt addition was omitted.

Fluorescence lifetime measurements were carried out on an Ortec 9200 photon counting nanosecond fluorometer. Excitation was selected by a Corning CS7-54 glass filter, emission by a CS3-68 filter. The excitation polarizer was set at vertical. Emission polarizer was set at 54.7° from the vertical. The time profile of the excitation was taken at the same wavelengths as those for the fluorescence decay in order to minimize errors due to variations in the time response of the RCA-8850 photomultiplier as a function of wavelength (Rayner et al., 1976). The extent of light scattering for the filter combination that was used was estimated to be about 2% of the fluorescence by using a Ludox colloidal sillica suspension that scatters light to the same extent as the samples. The data were analyzed by the method of moments procedure (Isenberg & Dyson, 1969) as described by Yguerabide (1972). It should be pointed out that more advanced methods for the analysis of fluorescence decay curves exist (McKinnon et al., 1977), and that the simple method of moments procedure may not yield the most accurate decay parameters. We do not believe, however, that there are gross errors in our results because of a number of internal checks within these measurements. As will be shown later, these include the linearity of the Stern–Volmer plots and the correspondence between the lifetime quenching measurements and the steady-state quenching measurements.

Steady-state fluorescence measurements were carried out on a Perkin-Elmer Hitachi MPF-4 spectrofluorometer. Fluorescence polarization measurements were made on a laboratory built instrument that records the polarization ratio

Table I: Fluorescence Decay Parameters of 1,5-IAEDANS-Labeled Actins[a]

material	A_1	τ_{o1} (ns)	A_2	τ_{o2} (ns)	χ^2/N
AEDANS-G-actin	0.957	17.25	0.043	33.30	8.35
urea + AEDANS-G-actin	1.0	13.63			27.3
EDTA + AEDANS-G-actin[b]	0.995	17.81	0.005		34.0
AEDANS-F-actin	1.0	19.45			4.85

[a] See Methods section for experimental conditions. A_1 and A_2 are the amplitudes of the major and minor decay components respectively; τ_{o1} and τ_{o2} are the corresponding lifetimes. All values were obtained from two exponential method of moments analysis. The reproducibility of the lifetime determinations were estimated to be 0.5 ns. χ^2/N is a parameter for judging the quality of the fit defined as $1/N\Sigma_{i=1}^{N}[F_c(t_i) - F_e(t_i)]^2/F_e(t_i)$ where N is the number of points, and F_c and F_e are the calculated and the experimental fluorescence decay, respectively. [b] Amplitude of minor component was too small for accurate determination of its lifetime.

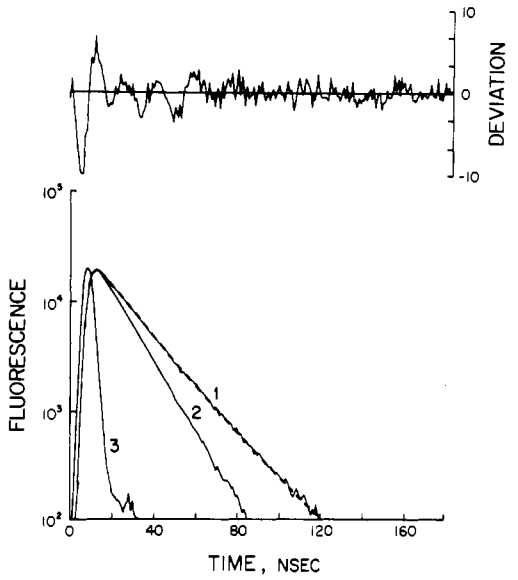

FIGURE 1: Fluorescence decay curves of (1) AEDANS-G-actin, (2) urea-denatured AEDANS-G-actin, (3) the excitation pulse. The dashed line superimposed on curve 1 is the calculated curve (F_c) by using parameters obtained from method of moments analysis. The top figure is the deviation between the calculated curve (F_c) and the experimental curve 1 (F_e), defined as $(F_c - F_e)/\sqrt{F_e}$. See Table I for compilation of lifetime results.

$R = I_{\parallel}/I_{\perp}$ directly. Viscosity measurements were made with a Cannon-Manning size 150 A554 semimicroviscometer. Quenching studies were carried out by adding small volumes of 6 M acrylamide. Appropriate corrections for dilution and acrylamide absorption were made for the steady-state measurements (Lehrer & Leavis, 1978). Unless otherwise noted, all studies were carried out at 25 °C, at a protein concentration of ~0.5 mg/mL, and labeling ratio of ~0.5 mol of label per mol of actin.

Results

Lifetime Studies. (See Table I for a compendium.) The fluorescence decay curve of AEDANS-G-actin plotted on a semilogarithmic scale clearly deviates from linearity (Figure 1). To first approximation we assumed that the decay was composed of two exponentials and analyzed the curve by the method of moments. The procedure yielded a major decay component of lifetime 17.3 ns that accounted for 96% of the total fluorescence, and a minor decay component of lifetime 33.3 ns accounting for the remaining 4%. Note that the fit

Table II: Fluorescence Quenching Rate Constants for 1,5-IAEDANS-Labeled Actins[a]

material	$k_q \times 10^{-7}$ (M^{-1} s^{-1}) major component	$k_q \times 10^{-7}$ (M^{-1} s^{-1}) minor component
AEDANS-G-actin	23.1 ± 0.4	6.5 ± 0.9
AEDANS-F-actin	13.5 ± 0.12	1.52 ± 0.06
EDTA + AEDANS-G-actin	22.3 ± 4.4	
AEDANS-F-actin (−TM)	13.5 ± 0.6[b]	
AEDANS-F-actin (+TM)	9.7 ± 0.6[b]	

[a] Unless otherwise noted all values were obtained from lifetime quenching data. The uncertainty values are standard deviations of the linear regression fits. [b] Values obtained from combination of lifetime and steady-state quenching measurements (see Discussion section). Uncertainties were estimated from the reproducibilities in K_{SV} (0.1 M^{-1}) and in τ_0 (0.5 ns).

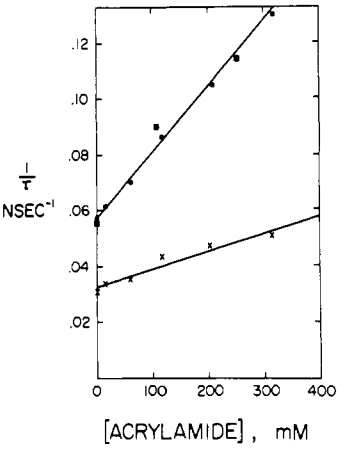

FIGURE 2: Stern-Volmer plots for major decay component of AEDANS-G-actin (●), minor decay component of AEDANS-G-actin (X), and major decay component of EDTA-denatured AEDANS-G-actin (■). See Table II for results.

was excellent, judging from the fact that the deviation between the experimental and calculated curves was small and random over more than four decades of decay (Figure 1, curve 1 and top panel). When the AEDANS-G-actin was denatured in 6 M urea, a single decay time of 13.6 ns was obtained, indicating that the heterogeneity in the fluorescence decay of native AEDANS-G-actin is not an intrinsic property of the label itself.

AEDANS-G-actin was also denatured by the addition of EDTA to 20 mM to remove the tightly bound divalent cations. The fluorescence decay curve (not shown here) was recorded after changes in the tryptophan fluorescence (Lehrer & Kerwar, 1972) had been completed. There appeared to be a decrease in the relative amplitude of the minor decay component, but the lifetime of the major decay component for EDTA-denatured AEDANS-G-actin was identical with that of native AEDANS-G-actin within experimental error.

The fluorescence decay of AEDANS-F-actin (not shown) was found to be nearly a single exponential of lifetime 19.5 ns.

Lifetime Quenching Studies. (See Table II for a compendium.) The fluorescence decay curves of AEDANS-G-actin at various acrylamide concentrations were obtained. Analysis by the method of moments showed that the decay curves are well represented by the sum of two exponentials at all acrylamide concentrations. The lifetimes of the two components were obtained and plotted according to the Stern-Volmer equation (eq 1) (Figure 2). Good linearity was obtained for both components, indicating that each of the two components is largely a pure exponential. The values of k_q

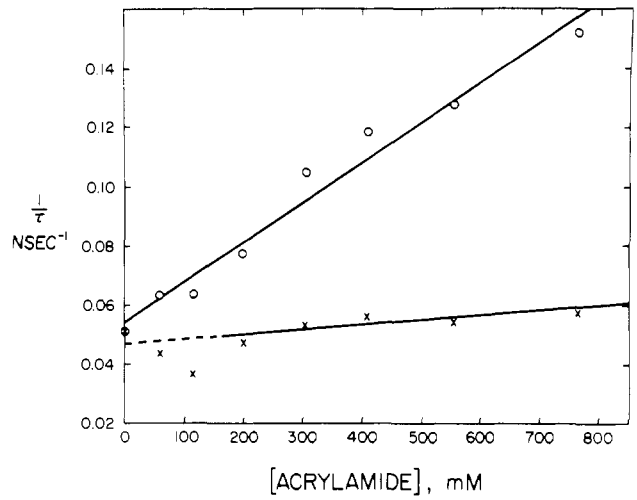

FIGURE 3: Stern–Volmer plots for major component of AEDANS–F-actin (O) and minor component of AEDANS–F-actin (X). The first three points for the minor component were omitted from the linear regression fit for reasons given in the Results section. See Table II for results.

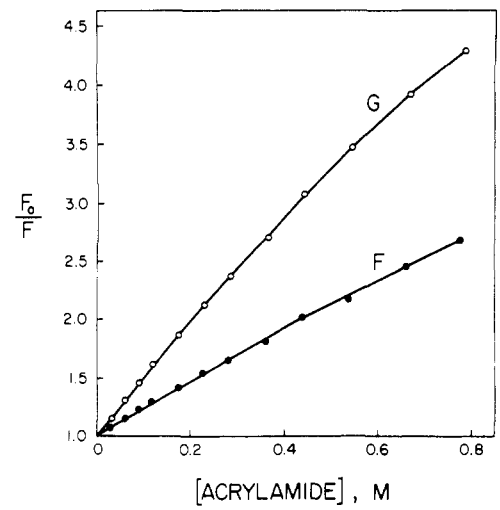

FIGURE 4: Steady-state intensity quenching curves for AEDANS–G-actin (O) and AEDANS–F-actin (●). λ_{ex} = 337 nm,; λ_{em} = 480 nm. Initial slopes were 5.05 and 2.51 M^{-1} for AEDANS–G-actin and AEDANS–F-actin, respectively. The reproducibility of these measurements was estimated to be 0.1 M^{-1}.

(obtained from the slopes of the Stern–Volmer plots) showed that the major component is 3.5 times more susceptible to quenching than the minor component.

k_q for the major component of EDTA-deuterated AEDANS–G-actin was also determined (Figure 2 and Table II) and found to be identical with that of native AEDANS–G-actin.

The lifetime quenching data (Figure 3) for AEDANS–F-actin were treated in the same manner as those for AEDANS–G-actin. Although the fluorescence decay of AEDANS–F-actin is nearly a single exponential in the absence of acrylamide, the presence of the minor component became revealed when acrylamide was present. It is apparent that, at zero acrylamide concentration, the amplitude of the minor component relative to that of the major component is too small to be detected by the method of moments procedure. The magnitudes of k_q (see Table II) show that, as for AEDANS–G-actin, the major component of AEDANS–F-actin is preferentially quenched by acrylamide; hence, the relative amplitude of the minor component is increased as acrylamide was added. Further, we believe that, although large enough to be detected, the amplitude of the minor component is still too small for reliable determinations of its lifetimes at low acrylamide concentrations. This is borne out by the observation that the first three points in the Stern–Volmer plot for the minor component of AEDANS–F-actin decreases (τ increases) with increasing acrylamide concentration, in apparent violation of the Stern–Volmer law (Figure 3, lower curve). For this reason, we excluded these points from the linear regression analysis from which k_q was determined. Inclusion of all the points resulted in an increase of 28% in the value of k_q.

Steady-State Quenching Studies. Figure 4 shows the intensity quenching plots (F_0/F vs. [Q]) for AEDANS–G- and F-actin. No upward curvature was detected for either curve, indicating that V is small. Instead, downward curvature was apparent for both curves, indicating the presence of multiple emission components. The initial slopes of the curves were determined to be 5.05 M^{-1} and 2.51 M^{-1} for AEDANS–G- and F-actin, respectively. Based on the lifetime studies we can expect the initial slopes to contain contributions from the major component and the minor component according to

$$IS = f_1(K_1 + V_1) + f_2(K_2 + V_2)$$

where the subscripts "1" and "2" refer to the major and minor components, respectively. We can show that, for both AEDANS–G-actin and AEDANS–F-actin, the contribution from the minor component is negligible by means of the following arguments. The magnitudes of f_1K_1 and f_2K_2 can be estimated using values of the lifetime (Table I) and the quenching rate constants (Table II). Thus, for AEDANS–G-actin

$$\begin{aligned} f_1K_1 &= f_1\tau_{o1}k_{q1} \\ &= (0.96)(17.3 \times 10^{-9}\ s)(23.1 \times 10^7\ M^{-1}\ s^{-1}) \\ &= 3.84\ M^{-1} \end{aligned}$$

Similarly we obtained $f_2K_2 = 0.09\ M^{-1}$. Thus f_2K_2 amounts to only 2% of f_1K_1 and can safely be ignored. Eftink & Ghiron (1976a) showed that the V parameter for an inaccessible emitter is generally smaller than that for an accessible one. Since we found that $k_{q2} < k_{q1}$ for AEDANS–G-actin, we can expect $V_2 < V_1$. Since $f_2 \sim 0.04f_1$, the magnitude of f_2V_2 cannot be more than 4% of f_1V_1 and can be ignored also. Hence, we can write

$$IS \simeq K_1 + V_1 \qquad (6)$$

where we have approximated f_1 as unity.

The same arguments can be made for AEDANS–F-actin. In fact, since f_2 is probably even smaller than f_1, and k_{q2} was found to be even smaller than k_{q1}, the arguments are even stronger in this case. No calculations were made because the values of f_2 and τ_{o2} were undeterminable.

The magnitudes of V_1 can now be estimated by using eq 6. For AEDANS–G-actin:

$$\begin{aligned} V_1 &= 5.05\ M^{-1} - 4.00\ M^{-1} \\ &= 1.05\ M^{-1} \end{aligned}$$

Similarly, for AEDANS–F-actin

$$\begin{aligned} V_1 &= 2.51\ M^{-1} - (19.5 \times 10^{-9}\ s)(13.5 \times 10^7\ M^{-1}\ s^{-1}) \\ &= -0.12\ M^{-1} \end{aligned}$$

Thus, whereas the magnitude of the V parameter for the major component of AEDANS–G-actin is about 50% of that for indole in water (Eftink & Ghiron, 1976b), it is equal to zero within experimental error for the major component of AEDANS–F-actin. This is consistent with the views of Eftink & Ghiron (1976a), who found that the extent of static

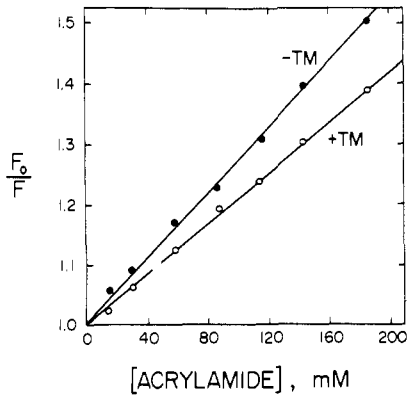

FIGURE 5: Intensity quenching curves for AEDANS–F-actin in the absence (●) and presence (○) of tropomyosin. Initial slopes (i.e., K_{sv}) are 2.64 ± 0.04 M^{-1} and 2.08 ± 0.002 M^{-1} in the absence and presence of tropomyosin, respectively. Uncertainty values were standard deviations of the linear regression fits. Reproducibility of the K_{sv} values was estimated to be 0.1 M^{-1}.

quenching is small for inaccessible sites, because quencher molecules are excluded from occupying the immediate vicinity of the emitter.

The major conclusion from the steady-state quenching studies is that for AEDANS–F-actin contributions from both static quenching and the minor component can be ignored, such that the initial slope of an intensity quenching plot for AEDANS–F-actin is to a good approximation equal to K_{sv} of the major decay component.

Tropomyosin Studies. Tropomyosin was added to AEDANS–F-actin at a molar ratio of 2 tropomyosin dimers (M_r = 66000) to 7 actin subunits. The final ionic composition was 100 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.2 mM ATP, 2 mM Hepes, pH 7.5. These are conditions under which tropomyosin is known to bind to F-actin (Eaton et al., 1975). Upon the addition of tropomyosin there was an increase in the fluorescence intensity of AEDANS–F-actin by a factor of 1.09, and a decrease in the initial slope from 2.64 M^{-1} to 2.08 M^{-1} (Figure 5). We calculated that for the major decay component of AEDANS–F-actin in the absence of tropomyosin

$$k_q = K_{sv}/\tau_0$$
$$= 2.64\ M^{-1}/19.5 \times 10^{-9}\ s$$
$$= 1.35 \times 10^8\ M^{-1}\ s^{-1}$$

That the value obtained here is in excellent agreement with the value obtained from the lifetime quenching studies (see Table II) highlights the reproducibility and internal consistency of these measurements. For AEDANS–F-actin in the presence of tropomyosin, we assumed that the lifetime increases by a factor of 1.09. This is justified because Hudson & Weber (1973) showed that the lifetime of the AEDANS moiety varies proportionally with its quantum yield. Thus, we obtain:

$$k_q = 2.08\ M^{-1}/21.3 \times 10^{-9}\ s$$
$$= 9.7 \times 10^7\ M^{-1}\ s^{-1}$$

It is apparent that k_q for the major labeling site of AEDANS–F-actin decreases by 28% when tropomyosin is present.

Effect of Acrylamide. The possible effect of acrylamide on the polymerizability of AEDANS–G-actin was examined by monitoring the fluorescence polarization. Like the viscosity, the steady-state fluorescence polarization of AEDANS–F-actin is characteristically higher than that of AEDANS–G-actin and can be used to assess the extent of polymerization. We found that, at room temperature, the polarization ratio ($R = I_{\parallel}/I_{\perp}$) of AEDANS–G-actin was 1.35, both in the absence and presence of 1 M acrylamide. Upon addition of KCl to

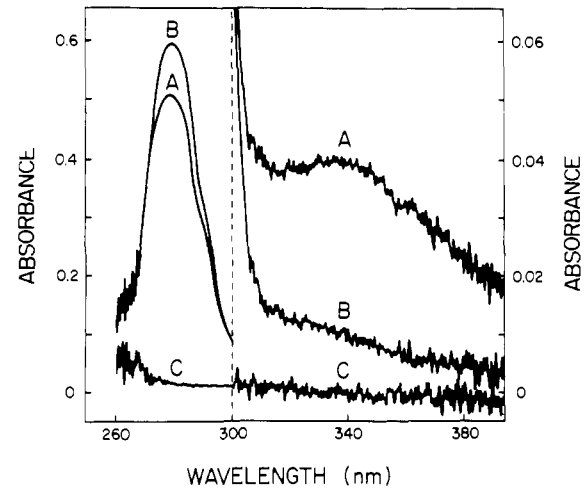

FIGURE 6: Absorption spectra of (A) AEDANS–G-actin, (B) AEDANS–MalNEt–G-actin, (C) buffer base line. Left-hand scale is for the spectrum below 300 nm. Right-hand scale is for the spectrum above 300 nm. AEDANS–MalNEt–G-actin contains 1.0 mol of MalNEt/mol of actin.

50 mM and $MgCl_2$ to 2 mM, R reached a maximum of 1.8 at roughly the same rate both in the absence and presence of 1 M acrylamide. After storage in 1 M acrylamide for 12 h, there was no decrease in R for AEDANS–F-actin. Thus the presence of acrylamide has no effect on the polymerizability of AEDANS–G-actin and does not break down the integrity of the AEDANS–F-actin filaments.

Blocking Studies with MalNEt. AEDANS–MalNEt–G-actin and a control sample of AEDANS–G-actin were prepared as described in the Methods sections. As shown in the absorption spectra of the two samples (Figure 6), there is very little AEDANS absorption at 337 nm for the AEDANS–MalNEt–G-actin sample, indicating that very little label was incorporated. The labeling ratio for the AEDANS–G-actin sample was determined from A_{337} and A_{290} (see Methods section) to be 0.63 mol of label/mol of actin. The labeling ratio for the AEDANS–MalNEt–G-actin sample was determined by comparing the fluorescence intensities of the two samples after appropriate corrections for the difference in protein concentration had been made. This was found to be 0.08 mol of label/mol of actin, which amounts to 13% of the labeling ratio for AEDANS–G-actin. It is clear that preincorporation of MalNEt into G-actin substantially reduces further incorporation of 1,5-IAEDANS, suggesting that the two reagents react with the same residue in G-actin.

Effect of Viscosity. It is well known that the polymerization of actin is accompanied by a large increase in the bulk viscosity. In theory, one can expect the quenching process to be viscosity dependent since the quenching rate constant is proportional to the translational diffusion coefficient of the quencher, which in turn is inversely proportional to the *microviscosity* (or local viscosity) of the medium. We examined the question of whether the microviscosity of an actin solution changes with polymerization by utilizing the Ans–apomyoglobin complex as a "microviscosity probe". It had been shown that the rotational correlation time of Ans–apomyoglobin increases with the microviscosity according to the Stokes–Einstein equation for rotational diffusion (Tao, 1969), and the steady-state fluorescence polarization increases with the microviscosity according to Perrin's Law (Stryer, 1965). Thus, by measuring the fluorescence polarization of Ans–apomyoglobin in the presence of G-actin and/or F-actin, one can quantitatively determine the microviscosity. Figure 7 shows recorder tracings of the polarization ratio R for Ans–apo-

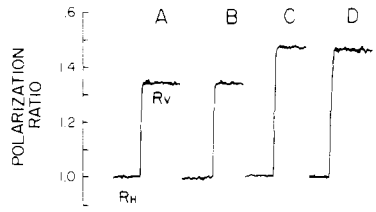

FIGURE 7: Recorder tracings of the polarization ratio at 28.5 °C for Ans–apomyoglobin (3.4 μM in both Ans and apomyoglobin) in the presence of (A) 4.5 mg/mL G-actin, relative viscosity $\eta = 1.027\ \eta_0$ (η_0 is the viscosity of water at 28.5 °C), (B) sample A with added KCl to 50 mM and $MgCl_2$ to 2 mM, $\eta = 7.08\ \eta_0$, (C) sample B with added sucrose to 20%, (D) Ans–apomyoglobin in 20% sucrose alone, $\eta = 1.470\ \eta_0$. The lower traces (marked R_H) represent the ratio I_{hv}/I_{hh}, where I_{hv}, for example, is the polarization component with the polarizer set in the horizontal position and the analyzer set in the vertical position. R_H was set to unity as calibration. The upper traces (marked R_V represent the polarization ratio $R = I_{vv}/I_{vh} = I_{\parallel}/I_{\perp}$.

Table III: Dependence of the Viscosity and the Quenching Constant K_{sv} on the Concentration of 1,5-IAEDANS-Labeled F-actin[a]

[AEDANS–F-actin] (mg/mL)	η_{sp}	η	K_{sv} (M^{-1})
0.111	0.056	$1.056\eta_0$	2.51
0.222	0.111	$1.111\eta_0$	2.30
0.444	0.221	$1.220\eta_0$	2.48

[a] Temperature was 25 °C. η_{sp} is the specific viscosity; $\eta_{sp} = (\eta - \eta_0)/\eta_0$. η_0 is the viscosity of water at 25 °C. Reproducibility of K_{sv} values was estimated to be 0.1 M^{-1}.

myoglobin in the presence of G-actin, F-actin, F-actin and 20% sucrose, and 20% sucrose alone. It is clear that there is no change in the polarization (and, hence, in the microviscosity) when G-actin is converted to F-actin. On the other hand, the bulk viscosity as measured by capillary flow increased 6.9-fold. Only the addition of sucrose caused an increase in the polarization ratio. The increase in R is the same in 20% sucrose alone as in 20% sucrose and F-actin, showing that the presence of the F-actin does not contribute to the microviscosity. These results clearly show that there is no increase in the microviscosity accompanying the polymerization of actin.

The effect of viscosity on the quenching process was also studied by varying the concentration of AEDANS–F-actin. As shown in Table III, while the kinematic viscosity increased by a factor of 1.16 with a fourfold increase in concentration (the specific viscosity η_{sp} increased linearly with concentration as expected), there was no detectable decrease in K_{sv} of the major component within experimental error. Both this result and the Ans–apomyoglobin result verify the notion that in a F-actin solution the quencher molecules diffuse freely in the vast aqueous space between the actin filaments and experience no additional viscous drag over that of the solvent medium.

Discussion

Location of the Labeling Sites. Since it is known that the fluorescence of the AEDANS moiety is sensitive to the polarity of the environment (Hudson & Weber, 1973), it is reasonable to associate the two decay components with two labeling sites of different polarities. Various considerations strongly suggest that the major decay component is associated with a major labeling site. There is much evidence showing that, of the five sulfhydryls in actin, cysteine-373 near the C terminus is extraordinarily reactive (Lusty & Fasold, 1969; Bridgen, 1972; Lehrer et al., 1972; Elzinga & Collins, 1975; Bender et al., 1976; Lin, 1978). In particular, Elzinga & Collins (1975) and later Bender et al. (1976) found that MalNEt labels G-actin

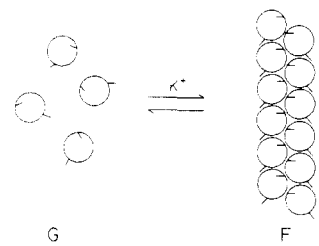

FIGURE 8: Schematic diagram for the interpretation of the quenching studies. The major labeling site in AEDANS–G-actin is exposed, while the minor site is buried. In AEDANS–F-actin, the major site becomes somewhat shielded, the minor site becomes deeply buried.

at cysteine-373 with 85–90% specificity. Our finding that MalNEt blocks the incorporation of 1,5-IAEDANS into G-actin by 87% (see Results section), therefore, indicates that 1,5-IAEDANS labels G-actin primarily at cysteine-373 also. Recently, Lin (1978) obtained similar results showing that dansylaziridine is primarily specific for cysteine-373.

The origin of the minor decay component is unclear. It was shown that iodoacetamide (Bridgen, 1972) and an azobenzene derivative of iodoacetamide (Lusty & Fasold, 1969) label actin at cysteines-10 and -256 as well as -373. Most likely, therefore, the minor decay component is associated with a labeling site (or a class of labeling sites) at either cysteine-256 or cysteine-10 or both. It is, however, difficult to rule out the possibility that the minor decay component emanates from a small amount of denatured material (although care was taken to remove such materials by ultracentrifugation), or from a minor conformational state that finds the label in a different environment even though the label is attached at only one site. Due to this ambiguity in its origin, we will not emphasize the properties of the minor decay component. In order to facilitate the ensuring discussions, however, we shall retain the concept that a "minor labeling site" is associated with the minor decay component, with the understanding that it may originate from other sources mentioned above.

Polarity and Steric Environments of the Sites. Since the fluorescence yield and, hence, the lifetime of the AEDANS moiety increases with decreasing polarity of the medium (Hudson & Weber, 1973), the lifetime data in Table I can be used to construct a sequence in *descending* order of polarity: (1) urea denatured AEDANS–G-actin; (2) major site of native and EDTA denatured AEDANS–G-actin; (3) major site of AEDANS–F-actin; and (4) minor site of AEDANS–G-actin. Similarly, the values of k_q shown in Table II allow us to construct a sequence in descending order of accessibilities: (1) major site of native and EDTA denatured AEDANS–G-actin; (2) major site of AEDANS–F-actin; (3) minor site of AEDANS–G-actin; and (4) minor site of AEDANS–F-actin. Note that, for those species that appear on both lists, the order of accessibilities is identical with the order of polarities (although the changes in the values of k_q are much larger than the changes in the lifetimes). This is consistent with the general view that an accessible site is polar in environment owing to the presence of water molecules, and an inaccessible site is nonpolar owing to the exclusion of water molecules.

Both our lifetime results and our quenching results suggest a model that is schematically represented in Figure 8: AEDANS–G-actin contains a major labeling site located at cysteine-373 and a minor labeling site (or class of labeling sites) of unknown location. Whereas the major site is considerably exposed, and probably lies on the surface of the actin subunit, the minor labeling site is substantially buried. Upon conversion to AEDANS–F-actin, both sites become less exposed, possibly due to direct shielding by amino acid residues

on neighboring actin subunits. The magnitudes of k_q (Table II) indicate that this shielding effect is more pronounced for the minor site than for the major site, suggesting that the major site is located near but not directly at the interface between successive actin subunits, while the minor labeling site probably lies on one of the interfaces and becomes deeply buried in the interior of the AEDANS–F-actin structure upon polymerization.

Effect of Tropomyosin. Our results show that the presence of tropomyosin decreases the accessibility of the cysteine-373 site in AEDANS–F-actin by 28%. It is possible that tropomyosin exerts this effect by binding to actin at a site remote from cysteine-373, but induces a change in actin to a conformation in which cysteine-373 is more buried. We found, however, that neither the lifetime nor the accessibility of the label attached at cysteine-373 is affected by EDTA denaturization, suggesting that this region of the actin molecule is not very sensitive to conformational changes. A more likely interpretation for this decrease in accessibility might be that the tropomyosin binds actin at a site sufficiently near cysteine-373 to directly shield the label from quenching. There is some indication that myosin also binds near cysteine-373 of actin, because the reactivity of actin sulfhydryl groups (Bailin & Barany, 1967), and, in particular, cysteine-373 (Lusty & Fasold, 1969) is suppressed by the binding of myosin. Duke et al. (1976) reported that the reactivity of actin cysteine-10 toward 1,5-IAEDANS is enhanced by the binding of myosin, but the residue has now been identified as cysteine-373 (R. Takashi, personal communication). It is possible that the chemical properties of 1,5-IAEDANS is such that the proximity of certain amino acid residues in myosin enhances its reactivity with cysteine-373. These findings, together with our results, suggest that both tropomyosin and myosin bind near cysteine-373 in actin. Thus, the observation of Eaton et al. (1975) that tropomyosin inhibits the ATPase activity of HMM can be attributed to competition between tropomyosin and myosin heads for the mutual binding site on actin.

It is of interest to recall the spin label work of Stone et al. (1970), who found that a maleimide spin label attached to actin (presumably at cysteine-373) became highly immobilized upon polymerization. Ohyashiki et al. (1974) found small changes in the fluorescence yield and lifetime of *N*-(1-anilinonaphthyl-4)maleimide attached to F-actin when tropomyosin was present. Recently, saturation transfer ESR work (Thomas et al., 1979) showed that a maleimide spin label attached to F-actin is slightly immobilized in the presence of tropomyosin. Tonomura et al. (1969) found that a maleimide spin label attached to actin in the troponin–tropomyosin–F-actin complex was sensitive to calcium concentrations, but these workers did not report the effect of tropomyosin alone on the mobility of the spin label. Tawada et al. (1978) reported that tropomyosin alone has no effect on the fluorescence anisotropy decay time of 1,5-IAEDANS-labeled F-actin, but this decay time is sensitive to calcium when both troponin and tropomyosin are present. Our findings are consistent with most of the work quoted above insofar as we found the accessibility of the 1,5-IAEDANS-labeling site in actin to be affected by polymerization and the presence of tropomyosin.

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