NANOSECOND FLUORESCENCE DEPOLARIZATION STUDIES ON ACTIN LABELED WITH 1,5-IAEDANS AND DANSYL CHLORIDE

Evidence for label flexibility

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1. Introduction

Fluorescence depolarization studies on the rotational relaxation behavior of G- and F-actin have been performed [1–5]. For G-actin, such studies yield information on the size, shape and aggregation state of the monomer. More interestingly, such studies on F-actin can yield information on the flexibility of the polymer. This property may be significant to the structure and function of the thin filaments, especially when it pertains to cooperative behavior related to calcium regulation of muscle contraction. While the results of these studies on G-actin are generally in agreement with each other, there are discrepancies in the results reported for F-actin. When labeled with ϵ-ADP, F-actin exhibited no detectable fluorescence depolarization within the time scale of the experiment, and the rotational correlation time was estimated to be >10 μs at 21°C [3]. Yet F-actin labeled with either didansyl cystine [4] or pyrene maleimide [5] exhibited measurable depolarization, yielding correlation times of the order to 500 ns at room temperature. Furthermore, correlation times obtained from other physical studies did not agree with the fluorescence depolarization results. For example, saturation transfer EPR studies on maleimide spin-labeled F-actin yielded a rotational correlation time of ~50 μs [6], while a translational correlation time >1 ms was obtained from quasi-elastic light scattering measurements [7].

It is disconcerting that such large discrepancies should exist between the various physical studies on the same system. In an effort to resolve some of the discrepancies, I have carried out nanosecond fluorescence depolarization measurements on G- and F-actin labeled with two other probes: 1,5-IAEDANS, and dansyl chloride. For G-actin, the observed correlation times are roughly consistent with the size and shape of the actin monomer. For F-actin, both labels yielded rotational correlation times of the order of 500 ns, in agreement with [4,5]. After due consideration of all the other pertinent findings, these new results indicate that the observed correlation times of about 500 ns correspond to flexibility of the covalent fluorescence labels at their attachment sites.

Abbreviations: 1,5-IAEDANS, *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)-ethylenediamine; AEDANS-G, 1,5-IAEDANS-labeled G-actin; AEDANS-F, 1,5-IAEDANS-labeled F-actin; dansyl-G, dansyl chloride-labeled G-actin; dansyl-F, dansyl chloride-labeled F-actin

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2. Experimental

2.1. *Protein labeling*

Rabbit skeletal actin was prepared as in [8]. AEDANS-G was prepared by incubating a 10-fold molar excess of label with a 2 mg/ml solution of G-actin at 4°C for 12 h. Excess label was removed from labeled protein using a Sephadex G-25 column. The amount of bound label was determined from the $A_{337\ nm}$, using $\epsilon = 6000\ M^{-1}\ s^{-1}$ [9]. Actin concentra-

tion was determined by $A_{290\,nm}$ using $\epsilon = 0.63$ $(mg/ml)^{-1}$ cm^{-1} [10], and M_r 42 300 [11]. Typically, labeling ratios of 0.5 mol label/mol actin were obtained.

AEDANS-F was obtained by polymerizing AEDANS-G in 50 mM KCl and 2 mM $MgCl_2$.

Dansyl-G was prepared as follows: dansyl chloride was first adsorbed onto Sephadex G-10 beads using essentially the method for adsorbing fluorescent dyes onto celite beads [12]. Dansyl chloride (10 mg) was added to Sephadex G-10 beads (100 mg) in acetone (100 ml). The mixture was stirred at room temperature for 30 min, then the acetone was removed by rotary evaporation. The 10% (w/w) dansyl–Sephadex beads are left behind as residue. Next, an appropriate amount of 10% dansyl–Sephadex beads was added to a 2 mg/ml solution of G-actin to obtain a 10-fold molar excess of dansyl chloride to actin. The mixture was stirred for 30 min at pH 8.5 and 22°C. The reaction was then quenched by adjusting to pH 7.0. The Sephadex beads were removed by centrifugation in a bench top centrifuge and the supernatant was chromatographed on a Sephadex G-25 column. The amount of protein-bound dansyl group was determined by the $A_{340\,nm}$, using $\epsilon = 3300$ M^{-1} cm^{-1} [13]. Labeling ratios of 0.5–1.0 mol dansyl/mol actin were usually obtained.

Dansyl-G so prepared could not be polymerized. Dansyl-F was prepared in the same manner as for dansyl-G, except that excess label was removed either by dialysis or by continuous replacement of buffer using the Millipore 'molecular separator'.

2.2. *Determination of anisotropy decay curves*

Anisotropy decay curves were obtained by following the procedure in [14]: the individual components of polarized decay were obtained by single photon counting. The anisotropy decay curves were calculated using the following equation:

$$A(t) = \frac{R \cdot I_{\parallel}(t) - I_{\perp}(t)}{R \cdot I_{\parallel}(t) + 2I_{\perp}(t)}$$

where $A(t)$ is the time-dependent anisotropy, $I_{\parallel}(t)$ and $I_{\perp}(t)$ are the parallel and perpendicular decay components normalized to the same area under the curves, and R is the steady-state polarization ratio:

$$R = \bar{I}_{\parallel}/\bar{I}_{\perp}$$

The steady-state polarization components $\bar{I}_{\parallel}$ and $\bar{I}_{\perp}$ were determined on the single photon counting apparatus by measuring the counting rates for each component.

It should be noted that this procedure does not take into account the fact that the excitation pulse has a finite width. The resultant systematic error in the rotational correlation time was estimated to be about 3 ns, which is comparable to the random error in these measurements.

3. Results

The anisotropy decay curves for dansyl-G and AEDANS-G are shown in fig.1. The corresponding curves for dansyl-F and AEDANS-F are shown in fig.2. Values of the rotational correlation times and steady-state anisotropy are summarized in table 1.

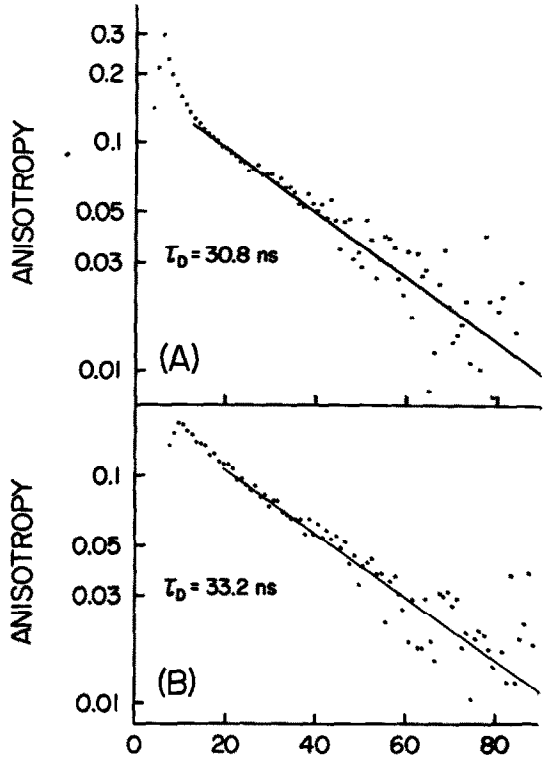

Fig.1. Anisotropy decay curves at 22.8°C for: (A) dansyl chloride-labeled G-actin; (B) 1,5-IAEDANS-labeled G-actin. Rotational correlation times were obtained from the slopes of best one exponential fits (solid lines).

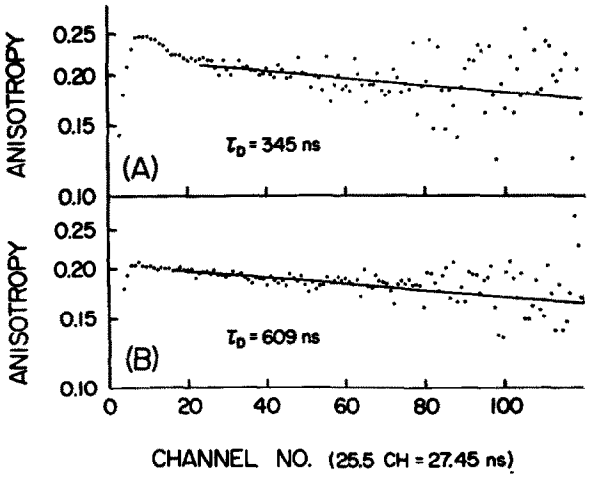

Fig.2. Anisotropy decay curves at 22.8°C for: (A) dansyl chloride-labeled F-actin; (B) 1,5-IAEDANS-labeled F-actin. Rotational correlation times were obtained from the slopes of best one exponential fits (solid lines).

The anisotropy decay curve for AEDANS-G showed little if any curvature in a semilogarithmic plot (fig.1B). The entire curve was fitted to a single exponential. Measurements on two preparations yielded an average correlation time of $\phi^{25°,w}$ = 29.2 ns (this value was adjusted from that at 22.8°C using the Einstein-Stokes equation and standard viscosity tables [15]). The uncertainty in these measurements was estimated to be about 5 ns.

Table 1
Rotational correlation times of fluorescence labeled actin[a,b]

Sample	$\phi^{25°,w}$ (ns)	$\bar{A}$
AEDANS-G	27.0, 31.3	0.104 ± 0.01
Dansyl-G	29.1, 30.3	0.112 ± 0.01
AEDANS-F	576, 605	0.189 ± 0.01
Dansyl-F	397, 326, 455	0.212 ± 0.01

[a] $\phi^{25°,w}$ is the rotational correlation time at 25°C in water. The values were calculated from the anisotropy decay times at 22.8°C using the Einstein-Stokes equation and standard viscosity tables [15]. The two values separated by commas represent measurements on two different labeled protein preparations. For dansyl-F, the first two values were measurements on samples for which excess label was removed by dialysis. The third was on a sample for which continuous buffer replacement was used to remove excess label

[b] $\bar{A}$ is the steady-state anisotropy measured at 22.8°C. $\bar{A}$ was calculated from the steady-state polarization ratio R using the equation $\bar{A} = (R-1)/(R+2)$

The anisotropy decay for dansyl-G shows a rapid decay superimposed on a slow decay (fig.1A). The slow decay corresponds to a rotational correlation time of $\phi^{25°,w}$ = 29.7 ns.

AEDANS-F shows a single very slow anisotropy decay corresponding to $\phi^{25°,w}$ = 590 ns (fig.2B). Due to the fact that the correlation times in these measurements are much longer than the fluorescence decay times of the label, the error in these measurements is probably as large as 200 ns.

Dansyl-F shows a similar long anisotropy decay of $\phi^{25°,w}$ = 393 ns. A fast component, however, is superimposed on this slow decay (fig.2A).

4. Discussion

4.1. *Rotational relaxation of labeled G-actin*

For compact globular proteins such as G-actin, the rotational correlation time should be a linear function of the molecular weight, provided that the partial specific volume and the degree of hydration do not vary much from one protein to another [16]. I have plotted the values compiled [14] and found that there is indeed a fair linear correlation. On this linear scale, the 29.7 ns correlation time obtained here for AEDANS-G corresponds to mol. wt 51 000, which is somewhat larger than the known M_r 42 300 for actin [11]. The value obtained here is also somewhat higher than $\phi^{25°,w}$ = 17.4 ns obtained in [2], and $\phi^{25°,w}$ = 17.9 ns obtained in [3]. Note that both measurements were made on ϵ-ATP-labeled G-actin, and that the values quoted here had been corrected from the values measured under the experimental conditions that the authors used. The larger value obtained here could be due to aggregation behavior, or to a slight elongation of the molecule in conjunction with a preferential orientation of the label [16,17]. Since no short component of anisotropy decay was found for AEDANS-G, it is evident that the label is rigidly bound within a 30 ns time scale.

It should be noted that 1,5-IAEDANS is specific for sulfhydryl groups. There is much evidence that of the 5 cysteine residues in actin, cysteine 373 near the

C-terminus is particularly reactive [18–21]. The fluorescence decay of AEDANS-G (not shown here) shows that 95% emission decays with a 17 ns lifetime. It is likely that this corresponds to the major labeling site at cysteine 373.

Dansyl chloride is an amino-directed probe, but may react with other groups as well [22]. When the labeling reaction was carried out in a pH 10 medium, as many as 12 dansyl groups could be incorporated into 1 mol actin. Even at labeling ratios <1.0, the fluorescence decay of dansyl-G is highly heterogeneous, containing at least 3 components of decay. Most likely there are multiple labeling sites in dansyl-G, with the label being less rigidly bound at some sites than at others. The flexible sites would give rise to a fast component of anisotropy decay, while the rigid sites would give rise to a component with a correlation time similar to that of AEDANS-G. It is possible, however, that the long component arises solely from restricted motion of the label at the flexible sites.

Thus the interpretation of the data for labeled G-actin is relatively straightforward. The major conclusions are that in AEDANS-G the label at the single labeling site is rigid within a 30 ns time scale. Dansyl-G contains multiple labeling sites, some of which are flexible, while possibly others are rigid within a 30 ns time scale.

4.2. *Anisotropy decay for labeled F-actin*

The results for F-actin are surprising. A molecule as large as F-actin is not expected to show any depolarization on this time scale. Yet both AEDANS-F and dansyl-F show substantial depolarization. The values of the correlation times obtained here are comparable to 411 ns at 21°C for didansyl cystine-labeled F-actin [4], and 560 ns at 25°C for pyrene maleimide-labeled F-actin [5]. ϵ-ADP–F-actin, on the other hand, shows no depolarization on the same time scale [3]. Other physical studies all place the correlation times above the microsecond range [6,7].

This observed depolarization could be due to flexibility in the polymer as a whole. Indeed, the quasi-elastic light scattering measurements [7] have suggested this possibility. However, the magnitude of the correlation time that these authors obtained is of the 1 ms order, more than 3 orders of magnitude higher than the values obtained here. It is not likely, therefore, that the two types of measurements were observing the same phenomenon.

As pointed out [4], it is also possible that the protein segment containing the labeling site is flexible. F-actin was spin-labeled with a maleimide derivative [6], probably at the same penultimate cysteine 373. These results suggest that such flexibility, if it occurs, takes place with a 50 μs correlation time. If flexibility occurs for the entire C-terminal segment of the protein, one would not expect the correlation times measured by different methods to differ by 2 orders of magnitude. Furthermore, dansyl chloride labels at several sites. Extensive depolarization is also observed, indicating that flexibility occurs at all sites. Since it is unlikely for a globular protein to possess several flexible segments, other mechanisms to account for the observed extensive depolarization should be considered.

A more likely interpretation and one that accounts for all the current information is that some of the probes might undergo flexing motion that is independent of the motion of the protein. The covalent probes 1,5-IAEDANS (this work), didansyl cystine [4], pyrene maleimide [5] and maleimide spin label [6] all possess at least 3 bonds that have complete torsional freedom. The presence of label flexibility had been suggested for eosin isothiocyanate-labeled ovalbumin [23], and for dansyl chloride-labeled membrane fragments [24]. It is likely that the observed anisotropy decay for the covalently labeled F-actins are due to flexing motion on the part of the label with respect to the more rigid F-actin framework.

Dansyl chloride also attaches covalently, but it is highly nonspecific, and probably attaches to ϵ-amino groups of several lysine residues. The side chains of each lysine residue may have different degrees of flexibility. This will serve to explain the presence of several correlation decay times in both dansyl-G and dansyl-F.

ϵ-ADP is a noncovalent probe that is known to be tightly bound at the nucleotide binding site of F-actin [2]. Label flexibility is not expected to occur in this system. It is, therefore, not surprising that no fluorescence depolarization was observed [3].

It might appear inconsistent that no rapid anisotropy decay attributable to label flexibility was observed for AEDANS-G. A possible explanation goes as follows: the data for AEDANS-F indicates

that the proposed label flexibility takes place on a 500 ns time scale. Since the rotational correlation time of AEDANS-G is more than an order of magnitude smaller (~30 ns), the label is effectively rigid on this time scale.

It should be cautioned that for all 3 types of measurements mentioned in this paper, no adequate theoretical treatment is available for describing such flexibility behavior. It is entirely possible that each method will yield a different correlation time for the same flexing motions, but not to such an extent that the results differ by 2–3 orders of magnitude.

5. Conclusion

It appears, therefore, that the discrepancies in the current literature on the rotational relaxation behavior of F-actin can be resolved if one takes into account the possibility that covalent labels can undergo flexing motion independent of the protein framework with an ~500 ns apparent rotational correlation time. This phenomenon of label flexibility is probably more widespread than heretofore recognized; this is because most fluorescence depolarization studies were carried out on monomeric globular proteins with rotational correlation times $<\sim$50 ns. Since label flexibility occurs with an ~500 ns apparent rotational correlation time, the phenomenon would not be detectable in such systems. For large protein aggregates with intrinsic rotational correlation times $\gg$500 ns, label flexibility would become apparent if no dominate the rotational relaxation behavior. Care should therefore be taken in the interpretation of anisotropy decay data for covalently labeled high molecular weight species.

It might be pointed out here that this observed label flexibility can be used as a sensitive monitor for the interaction of actin with the various contractile proteins. This will be extremely useful for unravelling the mechanism of energy transduction and calcium regulation in muscle. Studies using longer lifetime probes that will reduce the errors in such measurements are under way.

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