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TIME-DEPENDENT FLUORESCENCE DEPOLARIZATION AND LIFETIME STUDIES OF MYOSIN SUBFRAGMENT-ONE IN THE PRESENCE OF NUCLEOTIDE AND ACTIN

Robert Mendelson, Susan Putnam, and Manuel Morales

Cardiovascular Research Institute, University of California, San Francisco

Time-dependent fluorescence depolarization and lifetime studies have been made on myosin subfragment 1 to obtain information about mobility changes and dye environment changes when different nucleotides are added. Data are reported for static and actively hydrolyzing systems containing G- and F-actin. Preliminary data indicate that myosin labeled with the fluorophore 1, 5 IAEDANS[1] and treated with DTT preserves its actin-activated V_{max}. S1 prepared in this manner gives lifetime changes which are nearly identical for all systems studied. S1 labeling without DTT addition gives a pattern of lifetimes similar, though not identical to ESR work. Either type of labeling produces no observable change in the polarization decay, and we set an upper limit of 15% length change for the elongate S1. An unusually long fluorescence decay lifetime for the S1-Mg^{++} ATP-G-actin system is found which may indicate a new acto-S1 state stabilized by G-actin. The method for obtaining the bound fraction of S1's in the presence of actin is presented and applied to the S1-F-actin-Mg^{++} ATP system. Qualitative agreement is obtained with other methods.

INTRODUCTION

Recently the nanosecond fluorescence depolarization technique has been used to study the rotary mobility of myosin heads in the intact molecule and as proteolytic fragments (1). These studies have suggested that a hinge region exists between the S2 (rod portion) and the S1 (head) which acts like a universal joint, allowing the head to pivot during contraction (2). Additionally, these studies showed that S1 was more elongate than reported previously and thus that S1 is the major constituent of the cross-bridge seen in electron micrographs (3).

In this work we present preliminary results of our attempt to study the interaction of the S1 heads with various ligands and with actin. For myosin ATPase it has been suggested that (a) AMPPNP (4, 5) simulates the binding step of ATP; (b) the final stage of myosin hydrolysis must contain ADP with the system in its lowest free energy state; and (c) there is a rate-limiting step (6) which also is evident in steady-state spectroscopic experiments (7, 8). The aim of these experiments is to isolate these intermediates (and eventually others) with analogs and static experiments and then, by examining the steady-

[1] Abbreviations used: 1, 5 IAEDANS, N-(iodoacetylamino ethyl)-5-napthylamine-1-sulfonic acid; IAA, iodoacetamide; PEP, phosphoenolpyruvate; DTT, dithiothreitol; AMPPNP, adenyl imidodiphosphate; TES, N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid; ESR, electron spin resonance.

state hydrolyzing system (i.e. "+ ATP"), to find if there are additional spectroscopic intermediates that can be isolated. This strategy will also be applied to S1 + nucleotide + actin systems where, to date, only a rigor complex (S1 + F-actin) and possibly a rigor + products state have been seen. In these experiments the polarity of the dye environment is reported by the fluorescence lifetime and, independently and simultaneously, the rotational mobility is given by the polarization decay. The polarization decay gives information about possible shape changes in S1 + nucleotide systems and about the binding of S1 to actin.

METHODS

Myosin was prepared by the method of Tonomura et al. (9). S1 subfragments of myosin were prepared by a modification of the method of Lowey et al. (10) with the proteolysis conditions adjusted to give 75% or more of the mass of the heavy-chain fragment in the slowest-migrating peak on SDS gels.

Labeling of the S1 with the fast thiol-binding dye 1, 5 IAEDANS (11) was accomplished by either of two methods. In the first method (hereafter called Method I) the myosin was incubated for 18 hr at 4°C, pH 7, with 0.5 dyes per S1. This long incubation time ensured that the labeling had gone to completion. After the incubation, S1 fragments were produced and chromatographed on a Sephadex G-200 column. In the second method (hereafter called Method II) the chromatographed S1 fragments were labeled directly for 2 hr at 15°C, pH 7, with a starting molar ratio of 1.5 dyes per head present in the 20 μM S1 solution. Ca^{++}-activated ATPase indicated that approximately 0.3–0.6 moles of dye per mole of S1 were reacted. The reaction was stopped by lowering the temperature to 4°C and by adding dithiothreitol (DTT) to the solution to produce a final concentration of 2 mM DTT. The solution was immediately dialyzed against a storage buffer containing 0.5 mM DTT.

Actin was made by the technique described by Spudich and Watt (12).

Because experiments were performed on rapidly hydrolyzing systems, it was necessary to modify the fluorescence polarization decay apparatus used previously to allow accumulation of the largest possible number of photons during the data collection interval. Figure 1 shows the apparatus with improved lamp, pile-up rejection, and high-speed interface. Details of the system will be presented elsewhere.

Experiments were performed by making a control solution of 2 μM S1 containing 50 mM KCl, 50 mM TES, a PEP feeder system and, when appropriate, 0.5 mM DTT (see Table I). After a 9-min experimental interval a concentrated solution of the addition (nucleotide and/or actin) was added to produce a final concentration of 20μM of addition. Another 9-min counting interval followed. All experiments were conducted at 4°C.

Data analysis was performed by fitting the intensity and polarization decay data to a single exponential over a limited time interval. The changes in the 1/e times of polarization anisotropy decay (ϕ) and intensity decay (τ) were averaged from experiments made with different protein preparations.[2]

[2]The polarization anisotropy is defined as $r(t) \equiv \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)}$ and the fluorescence intensity is given by $I(t) = I_{\parallel}(t) + 2I_{\perp}(t)$. Here $I_{\parallel}(t)$ and $I_{\perp}(t)$ are the intensities parallel and perpendicular to the excitation polarization.

RESULTS

The results (Table I) obtained by using S1 made from labeled myosin (Method I) are more extensive and contain data from many preparations. However, we have found when using myosin prepared in this manner that the actomyosin V_{max} is significantly below that of unlabeled myosin. This loss of activity has also been observed for spin-labeled myosin when a spin label with similar iodoacetamide reactivity is employed (13, 14).

The observation of Seidel (14) that the V_{max} of spin-labeled myosin is maintained when DTT is added after labeling led us to the method of direct labeling of S1 with subsequent DTT addition (Method II). The data shown in Fig. 2 were obtained by removing aliquots of myosin during the labeling period, adding DTT to the aliquot, lowering its temperature, and measuring Ca^{++} and Mg^{++}-actin-activated ATPases. Although the degree of labeling varies from 0 to approximately 1 dye per head, as determined from Ca^{++} ATPase, the actin activation is independent of the degree of labeling. Myosin made by Method I gives an actomyosin ATPase V_{max} which is about two-thirds of that of unlabeled myosin.

Preliminary results of time-resolved fluorescence decay experiments with Method I indicate that the changes in ϕ are approximately the same for Method I or Method II, but the changes in lifetime are small and all positive – approximately 0.2 nsec. The ATPase of the Mg^{++}-S1-actin system was about 0.5 V_{max} for the 20 μM actin concentration used. G-actin experiments were not carried out with S1 made by Method II.

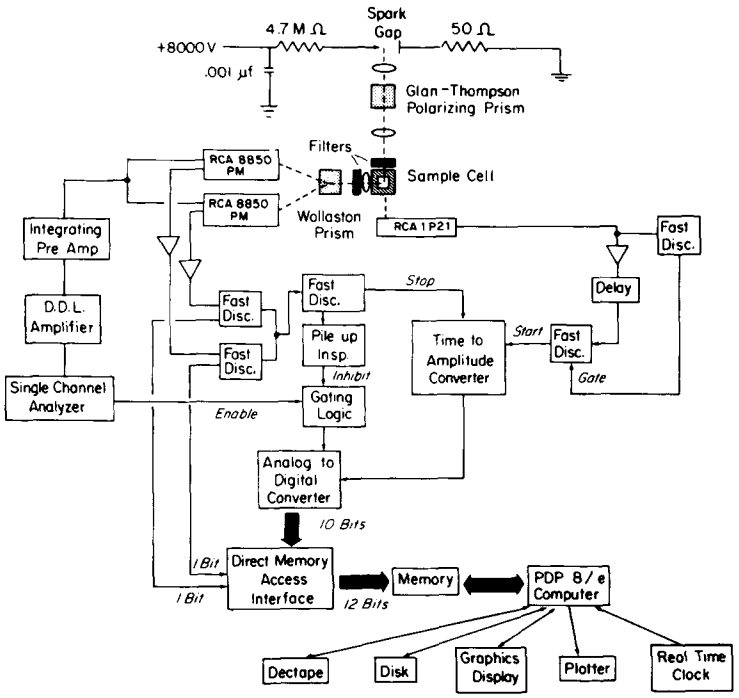

Fig. 1. Diagram of the optical and electronic apparatus used for simultaneous polarization decay and lifetime measurements.

TABLE I. Polarization Decay and Intensity Decay in Acto-S-1 Systems

Additions to S-1	Change in lifetime $\Delta\tau$ (nsec)	Change in polarization decay $\Delta\phi$ (nsec)	Number of trials
ATP	0.33 ± 0.02	8 ± 12	14
ADP + P_i	0.51 ± 0.04	17 ± 14	14
F-Actin + ATP (contraction)	0.29 ± 0.04	95 ± 13	10
F-Actin (rigor)	−0.28 ± 0.04	700 ± 300	8
F-Actin + ADP + P_i	−0.05 ± 0.05	700 ± 300	8
G-Actin + ATP	0.88 ± 0.06	140 ± 140	4
G-Actin + ADP + P_i (after hydrolysis)	−0.05 ± 0.07	500 ± 300	3
AMPPNP	0.54 ± 0.03	13 ± 16	6
AMPPNP + F · Actin	0.04	–	2
F-Actin + ATP (low ionic strength)	0.03	70	1

All errors are standard errors of the mean. All solutions except AMPPNP solutions contained 2 μM S1, 0.04 μM pyruvate kinase, 400 μM PEP, 50 mM TES, 150 μM MgCl, pH 7. The AMPPNP solutions were pH 8. Nucleotide or actin additions were in a final concentration of 20 M. The G-actin system contained 2 μM S1, 1 mM TES, 100 μM $MgCl_2$, 200 μM PEP, 0.02 μM pyruvate kinase, and 50 μM ATP. The $\phi \approx 200$ nsec for unligated S1.

Measurements of lifetime change and polarization decay change were also carried out on labeled myosin (without DTT) in 0.6 MKCl, pH 7. The lifetime increased (see Table II) but the ϕ value remained unchanged (approximately 450 nsec), indicating the dye is "immobilized" on a time scale much longer than the depolarization time of S1 in the presence of nucleotide. Thus, the ϕ's observed for S1-nucleotide systems are related only to the motion of the entire fragment.

DISCUSSION

The lifetime changes observed are measures of the effect of ligand or actin on the polarity of the dye environment. A comparison of ESR (with IAA spin label), intrinsic tryptophane fluorescence, and lifetime studies is shown in Table III. There is similarity between ESR mobility changes and extrinsic fluorescence lifetime changes with one notable exception. The rate-limiting step in myosin ATPase shows maximal change in ESR (and also intrinsic fluorescence) experiments but shows only intermediate change of the polarity of the dye's environment. There are indications that there is not a large change in the nature of the label site, as might be inferred from ESR work. The value obtained for the lifetime is about 21 nsec, which, from the work of Hudson and Weber (11), indicates the site is of a very hydrophobic nature and the changes in the availability of the solvent to the dye site are quite small. Hudson and Weber found that in going from a pure water to a pure ethanol solution the lifetime of free dye varies between 9 and 19 nsec.

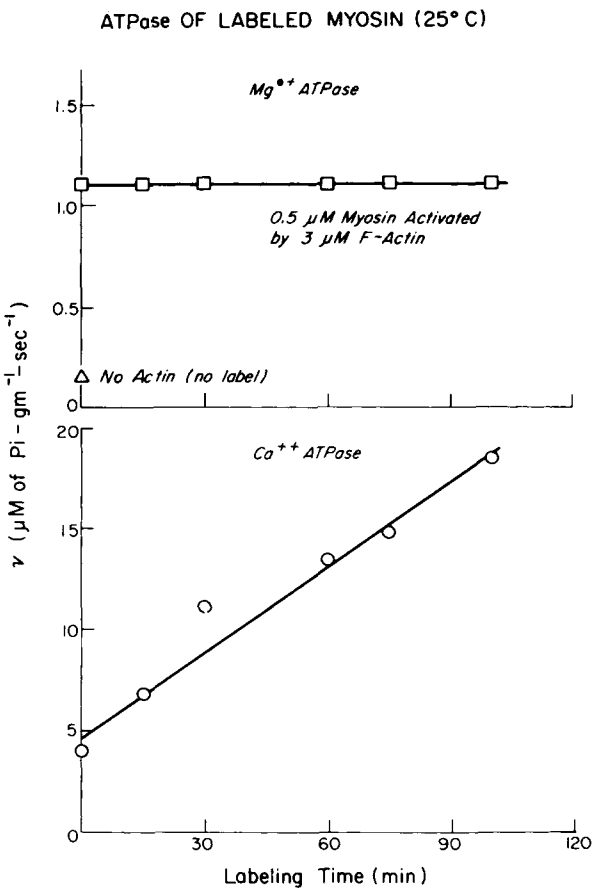

Fig. 2. Ca^{++} and Mg^{++} actin-activated ATPase of myosin labeled at 15°C with DTT immediately added to stop labeling and preserve the ATPase (Method II). Aliquots were taken at times shown on the abscissa and stored for less than 3 hr at 0°C with 2 mM DTT present. The myosin concentration is 0.5 μM and 1, 5 IAEDANS dye concentration is 1.5 μM. The myosin solution contained 50 mM TES, 50 mM KCl, pH 7. Actin-activated ATPase was measured with addition of 1 mM ATP and 1 mM $MgCl_2$. The lower curve shows the Ca^{++} ATPase which is related to dye binding; one dye per S1 activates approximately 5–6 fold. The upper curve is the Mg^{++} ATPase activated by 3 μM F-actin. When 18-hr labeling at 4°C is used with 0.5 dyes per head present, approximately one-third of the activity is lost at 3 μM actin concentration. At this actin concentration the ATPase is 75–100% of V_{max}. No observable loss of actin activity occurs with increasing amounts of dye bound to myosin.

The effect of adding DTT to restore the actin-activated V_{max} dramatically alters the pattern of lifetime changes. We are presently studying the possibility of a deleterious effect of DTT on protein with increasing time of incubation. If no effect is found, the ESR changes observed may also contain artifacts. These would be unobservable because the addition of DTT would also reduce spin label.

A singularly long lifetime is observed for the S1-G-actin-Mg^{++} ATP system. Because it is

TABLE II. Changes in Polarization Decay and Intensity Decay for Myosin

Addition	$\Delta\tau$ (nsec)	$\Delta\phi$ (nsec)	No. of trials
2 mM MgATP	0.3 ± 0.04	3 ± 10	8

Change in lifetime (τ) and polarization relaxation time (ϕ) of 1 μM myosin in presence of 2 mM MgATP. Myosin is in 0.6 M KCl, 50 mM TES, pH 7, 4°C.

TABLE III. Changes in Spin-Label Mobility, Extrinsic Dye Lifetime, and Intrinsic Fluorescence Intensity with Addition of Ligands and/or Actin

ESR (increasing mobility downward)	1, 5 IAEDANS fluorescence (increasing lifetime downward)	Intrinsic fluorescence (increasing intensity downward)
F-actin	F-actin	
F-actin + ADP	F-actin + ADP	none
none	none	AMPPNP
AMPPNP or ADP	ATP	ADP
ATP	AMPPNP or ADP	ATP

Observed changes in low-field spin-label mobility, 1, 5 IAEDANS fluorescence lifetime, and intrinsic fluorescence lifetime with addition of ligand and/or actin. From references 7, 8, 13 and this work. No DTT was used.

longer than observed with any other intermediate it may be indicating the presence of a new actomyosin intermediate. This would be expected if acceleration of ATPase by actin required the presence of more than one monomer to impose directionality for the head rotation. Ionic strength and pH effects cannot be ruled out because efforts to obtain adequate controls, at the very low ionic strength required to maintain a high fraction of G-actin, have been unsuccessful to date. No results with S1 made by Method II are available.

The absence of observable change of the ϕ value in the presence of nucleotide or analog indicates that delocalized changes in S1, during the detached phase of the contraction cycle, are small. An upper limit on the change in axial ratio, ρ, of an assumed prolate ellipsoid of revolution, was estimated utilizing the results of Belford et al. (15). By setting restrictions on the maximum length (200 Å) of S1 we have resticted the set of possible dye orientations (cf. ref. 1). Exhaustive calculations of ϕ values for different orientations and axial ratios indicated that for $\phi \approx 200$ nsec and ρ in the range 3 to 4.5,

$$\frac{\Delta_\rho}{\Delta_\phi} \approx 1.3 \times 10^{-2} \text{ nsec}^{-1}$$

Assuming a constant volume protein, we calculate that the change in the major axis of S1 is less than 8% in the presence of ATP for Method I S1. Method II S1 yields less than 10% change from more limited data. In both cases the counting statistics are the ultimate limiting factors. For other nucelotides the change in length is less than 15%. All limits are for 5% confidence intervals.

The formation of a rigor complex is seen for S1-actin and S1-actin-Mg^{++}ADP-P_i systems. The deviation from $\phi \to \infty$ may be indicative of a dynamic equilibrium between

S1 and actin as longer ϕ values have been observed (1).

The S1-F-actin-Mg^{++} ATP system ϕ values allow one to estimate an upper limit on the fraction of heads bound to actin during steady-state hydrolysis. If the lifetimes are essentially identical, as with S1 made by Method II, the polarization functions are additive and the polarization anisotropy for the steady-state system is written as

$$r(t) = \alpha r(0)e^{-t/\phi_1} + \beta r(0)e^{-t/\phi_2}$$

where $\alpha + \beta = 1$. For early times the experimental curve may be fitted to a single exponential over a limited range. Averaging over this range by time integration of both sides of the above equation and allowing $\phi_1 \rightarrow \infty$ gives a value for the ratio of bound to free S1 (α/β). Making approximate corrections for the fact that, at the actin concentrations used, we deviate from V_{max} conditions, and considering a 5% confidence interval, we find from Method I and II data (ignoring $\Delta\tau \neq 0$ in the former) that less than 35% of the heads are bound to actin. It should be noted that this does not allow for any increase in the microviscosity due to actin. If corrections could be made for this effect, the limit of the fraction bound would be lowered.

These results are in agreement with the kinetics work of Eisenberg et al. (16), who suggest that a small bound fraction indicates the presence of actin-binding chemical intermediate of myosin which has not, as yet, been observed spectroscopically.

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