Motion of Myosin Cross-Bridges in Skeletal Muscle Fibers Studied by Time-Resolved Fluorescence Anisotropy Decay†

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ABSTRACT: The time-resolved fluorescence polarization anisotropy signal has been measured from fluorescent-labeled myosin cross-bridges in single glycerinated muscle fibers in the relaxed and rigor states. In one experimental configuration, the polarization of the excitation light and the fiber axis are aligned, and the anisotropy is sensitive to rotational motions of the probes about axes other than the fiber axis. The rotational correlation times are ~1000 ns for relaxed fibers and >7000 ns for rigor fibers. In another experimental configuration, the excitation light polarization is perpendicular to the fiber axis, and its propagation vector has a component parallel to the fiber axis so that the anisotropy is sensitive to probe rotational motion about different axes, including the fiber axis. In this configuration, the rotational correlation times are ~300 ns for both relaxed and rigor fibers. The theory of rotational diffusion in a potential described in a related paper [Burghardt, T. P. (1985) *Biophys. J.* (in press)] is applied to the relaxed fiber data.

The angular distribution of myosin cross-bridges in skeletal muscle fibers is under investigation because the cross-bridges may participate in muscle contraction by rotating while attached to the actin filament (Huxley & Simmons, 1971; Morales et al., 1982). The orientation of cross-bridges in fibers has been investigated by using extrinsic probes attached to specific sites on the cross-bridges (Burghardt et al., 1983; Borejdo et al., 1982; Wilson & Mendelson, 1983; Thomas & Cooke, 1980). These probes are attached primarily to a single SH moiety (SH-1) of myosin subfragment 1 (S-1) in the fibers (Takashi et al., 1976; Borejdo & Putnam, 1977). In these techniques, the instantaneous distribution of probe orientations is measured while the muscle fiber is immersed in various solutions that induce known physiological (static or dynamic) states. Considerable effort has been expended to show what conclusions about cross-bridge orientation are justified on the basis of information about the probe orientation (Burghardt, 1984; Mendelson & Wilson, 1983).

Dynamical orientation measurements on cross-bridges have spanned a large time domain. Fluctuations in the polarized fluorescence from labeled cross-bridges have been used to examine rotational correlation times for the cross-bridges in the time domain of 10^{-3}–1 s (Borejdo et al., 1979). These experiments showed that cross-bridges undergo slow rotational motion in contracting muscle fibers. Saturation transfer electron spin resonance and phosphorescence polarization anisotropy decay have been used to investigate the rotational correlation times for cross-bridges in relaxed synthetic myosin filaments and in myofibrils in the time domain of 10^{-6}–10^{-3} s (Thomas et al., 1980; Barnett & Thomas, 1984; Eads et al., 1984). Time-resolved fluorescence polarization anisotropy decay (TRFAD) has been used to investigate the rotational correlation times, in the time domain of 10^{-9}–10^{-6} s, of subfragment 1 and of heavy meromyosin in solution (Mendelson et al., 1973; Mendelson & Cheung, 1978). TRFAD studies on cross-bridges in myofibrils have previously indicated essentially no motion in this time domain (Mendelson & Cheung, 1976).

We have applied the TRFAD technique to single glycerinated muscle fibers in which the cross-bridges are specifically labeled at the highly reactive SH-1 group with a fluorescent probe. In one experimental configuration, the polarization of the excitation light and the fiber axis are aligned, and the anisotropy is sensitive to rotational motions of the probes about axes other than the fiber axis. The rotational correlation times are ~1000 ns for relaxed fibers and >7000 ns for rigor fibers. In another experimental configuration, the excitation light polarization is perpendicular to the fiber axis, and its propagation vector has a component parallel to the fiber axis so that the anisotropy is sensitive to probe rotational motion about different axes, including the fiber axis. In this configuration, the rotational correlation times are ~300 ns for both relaxed and rigor fibers.

The theory for time-resolved fluorescence polarization of ordered assemblies, described in a related paper (Burghardt, 1985), can be used to interpret the data from relaxed fibers. In this theory, it is assumed that the cross-bridges are undergoing rotational diffusion in a potential. For probe motion about axes other than the fiber axis, the relaxed fiber data indicate rotational diffusion constants of $D_1 = 1.25 \times 10^5\ s^{-1}$ and $D_3 = 3 \times 10^5\ s^{-1}$ for the symmetrical cross-bridge molecule. In this configuration, the rigor cross-bridges have essentially an infinite rotational correlation time, indicating that the cross-bridge is immobilized for rotation about axes perpendicular to the fiber axis by its attachment to actin.

For probe motion about the fiber axis, both relaxed and rigor cross-bridges have relatively fast rotational correlation times. The decrease in the relaxed cross-bridge correlation time from that about the perpendicular axis of rotation is predicted by our theory (Burghardt, 1985). In the rigor state, because the cross-bridge is tightly bound to the actin filament, the rotational mobility is probably due to flexibility either in the

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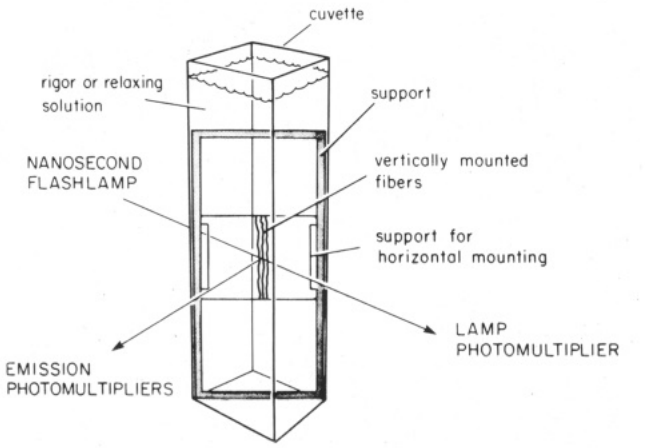

FIGURE 1: Sample holder. A 1.4 cm × 3.0 cm rectangular support fits diagonally in a standard quartz fluorescence cuvette filled with rigor or relaxing solution. Three bundles of one to five fluorescent-labeled fibers are mounted horizontally or vertically on the support. The cuvette is placed in a time-resolved fluorescence anisotropy instrument.

cross-bridge or in the actin filament, or in both.

Materials and Methods

Solutions. Rigor solution was 80 mM potassium chloride, 5 mM magnesium chloride, 2 mM ethylene glycol bis(tetraacetic acid), and 5 mM sodium phosphate, pH 7.0. Relaxing solution had the same composition, except that ATP was added at 4 mM.

Muscle Fibers. Rabbit psoas muscle fibers were obtained as previously described (Borejdo et al., 1979) and kept in a relaxing water/glycerol solution at −15 °C for up to several weeks. Subfragment 1 of myosin in the fiber was labeled with 5-[[[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid (1,5-IAEDANS; Molecular Probes, Junction City, OR) as previously described (Borejdo et al., 1982). When this procedure is used, ~86% of the 1,5-IAEDANS is covalently attached to a single SH moiety of myosin subfragment 1 (Borejdo & Putnam, 1977). After being labeled, the fibers were transferred to rigor solution, and two or three bundles of approximately five fibers each were mounted vertically or horizontally on a 1.4 cm × 3 cm rectangular support made to fit diagonally in a standard fluorescence cuvette (see Figure 1).

Fluorescence Anisotropy Decay Measurements. Fluorescence anisotropy decay was measured by using an instrument consisting of a nanosecond flash lamp, dual photomultipliers for polarized fluorescence detection, a photomultiplier for lamp detection, and pulse-counting electronics (including a time to amplitude converter and a pile-up rejection device) interfaced to a PDP8 minicomputer (Mendelson et al., 1973, 1975). Fluorescence was excited with vertically polarized light of wavelengths <370 nm and collected (at 90°) at wavelengths >450 nm. In one experimental configuration, the fiber was mounted vertically so that the excitation light polarization is parallel to the fiber axis (Figure 1). In another experimental configuration, the fiber was mounted horizontally and diagonally across the cuvette, so that the excitation light polarization is perpendicular to the fiber axis and the propagation vector has a component along the fiber axis (Figure 1). All experiments were done at room temperature, within 1 h after the fiber was mounted. The shape of the lamp pulse was measured by collecting excitation light scattered from oyster glycogen (Torgerson, 1984). The relative fluorescence collection efficiency of the two photomultipliers was estimated by collecting fluorescence from a solution of free 1,5-IAEDANS.

Data Analysis. For an isotropic sample and an infinitely sharp lamp pulse, the total fluorescence intensity, $F(t)$, is given by $F_\parallel(t) + 2F_\perp(t)$, where $F_\parallel(t)$ and $F_\perp(t)$ are the fluorescence intensities emitted parallel and perpendicular to the polarization of the excitation light. $F(t)$ is a sum of exponentials with lifetimes and amplitudes denoted by τ_i and f_i such that

$$F_\parallel(t) + 2F_\perp(t) = F(t) = \sum_i f_i \exp(-t/\tau_i) \qquad (1)$$

The different terms in eq 1 describe different populations of fluorescent molecules with different lifetimes.

For an anisotropic sample and an infinitely sharp lamp pulse, $F_\parallel(t) + 2F_\perp(t)$ is not the total intensity $F(t)$ but in general contains terms that depend on the fluorescence anisotropy relaxation rates. For a single population of molecules (Burghardt, 1985)

$$F_\parallel(t) + 2F_\perp(t) = F(t)[\sum_j \alpha_j \exp(-E_j t)] \qquad (2)$$

Parameters α_j and E_j are determined by the potential in which the probes diffuse, and the diffusion tensor. In our convention, the rate E_1 is always equal to 0. Thus, if the rates E_j with $j > 1$ are much slower than the time scale of interest (the largest significant lifetime), or if the coefficients α_j with $j > 1$ are small, then the infinite sum in eq 2 is approximately equal to α_1, and $F_\parallel(t) + 2F_\perp(t)$ is proportional to the fluorescence intensity, $F(t)$. For vertically mounted fibers in the rigor state, the rates E_j with $j > 1$ are very slow, and $F_\parallel(t) + 2F_\perp(t)$ is proportional to $F(t)$. For vertically mounted fibers in the relaxed state, we have used data from static polarization experiments (Wilson & Mendelson, 1983) and varied the diffusion constants until the calculated relaxation rates E_j agree with the experimentally measured relaxation rates. This calculation simultaneously yields α_j. We thus found that the time-dependent terms with $j > 1$ in the sum of eq 2 contribute less than 1% of the total sum. For horizontally mounted fibers in either the relaxed or the rigor state, the experimentally determined values of $F_\parallel(t) + 2F_\perp(t)$ have the same decay times and amplitudes as for vertically mounted fibers (see Results). We thus assume that the sum in eq 2 is nearly time independent also for the horizontal configuration.[1]

Because $F_\parallel(t) + 2F_\perp(t)$ is either proportional to or equal to $F(t)$ for all of the data, the standard method of separating fluorescence lifetimes from relaxation times in anisotropy data analysis can be applied. In this method, the anisotropy $r(t)$, obtained from the measured values of $F_\parallel(t)$ and $F_\perp(t)$, is an infinite sum of exponentials with rates E_j and amplitudes denoted by r_j (different from α_j), where

$$r(t) \equiv [F_\parallel(t) - F_\perp(t)]/[F_\parallel(t) + 2F_\perp(t)] = \sum_j r_j \exp(-E_j t) \qquad (3)$$

Parameters r_j and E_j are known functions of the potential in which the probes diffuse, and the diffusion tensor [see eq 27 of Burghardt (1985)].

When more than one population of fluorescent probes is present in the system, the observed anisotropy decay is a weighted average of anisotropies that are attributed to the individual probe populations. When there are two probe

[1] This argument presumes we do not attribute any of the very fast relaxation times observed (~10 ns, see Results) to cross-bridge motion. This is a reasonable assumption given the size of the cross-bridge.

Table I: Fluorescence Anisotropy Decay Data[a]

configuration	r_1	r_2	$1/E_1$ (ns)	$1/E_2$ (ns)
vertical				
relax	0.05 ± 0.02	0.25 ± 0.05	7.0 ± 5.0	1000 ± 200
rigor	0.04 ± 0.02	0.27 ± 0.06	5.0 ± 3.0	>7000
horizontal				
relax	0.04 ± 0.02	0.15 ± 0.03	5.0 ± 2.0	340 ± 70
rigor	0.025 ± 0.006	0.07 ± 0.04	10.0 ± 9.0	300 ± 200

[a] Shown are the mean ± the standard deviation of the amplitudes (r_i) and decay times (E_i) of fluorescence anisotropy decay data fitted with a sum of two exponentials. Each number represents an average over at least five experimental curves obtained from independently prepared fibers.

populations with fractional intensities of a and b ($b = 1 - a$), the observed anisotropy is, from eq 2 and 3

$$r(t) = \frac{a \exp(-t/\tau_a) r_a(t) + b \exp(-t/\tau_b) r_b(t)}{a \exp(-t/\tau_a) + b \exp(-t/\tau_b)} \quad (4)$$

where τ is the fluorescence lifetime and $r_{a(b)}(t)$ is the anisotropy decay for the individual a (or b) probe populations. Given that $\tau_a > \tau_b$ and $a > b$, we can approximate eq 4 with

$$r(t) = (1 - b^2) r_a(t) + [b(1 + b) r_b(t) - b(1 - b) r_a(t)] \times \exp[-t(1/\tau_b - 1/\tau_a)] - b^2 r_b(t) \exp[-2t(1/\tau_b - 1/\tau_a)] \quad (5)$$

From eq 5, we see that if $\tau_a \gtrsim 2\tau_b$, only $r_a(t)$ will contribute to $r(t)$ at times longer than the lifetime τ_a. This implies that long relaxation times of $r_a(t)$ can be observed from the signal $r(t)$.

Deconvolution from the finite-width lamp pulse was done by an iterative reconvolution nonlinear least-squares method (Grinvald & Steinberg, 1974; Badea & Brand, 1979; Torgerson, 1984). Goodness of fit was evaluated from the value of χ^2, visual inspection of the difference between experimental and theoretical curves (the residual), and visual inspection of the autocorrelation of residuals (Badea & Brand, 1979).

The fluorescence lifetime and anisotropy data were each well fitted with a sum of two exponentials corresponding to four free parameters: two amplitude factors and two decay times. One of the exponentials is attributed to probe motion from nonspecific probes in the muscle fiber; the other is attributed to cross-bridge properties (see Results).

Results

The fluorescence lifetime data, analyzed by using eq 1, were well fitted with a sum of two exponentials for both relaxed and rigor fibers and in both experimental configurations. The probes in rigor fibers (vertical and horizontal configurations) have lifetimes of 9.2 ± 0.6 and 20.1 ± 0.1 ns, with relative intensities of 0.16 ± 0.02 and 0.84 ± 0.02, respectively. In relaxed fibers (vertical and horizontal configurations), the probes have lifetimes of 11.0 ± 2.0 and 20.9 ± 0.4 ns, with relative intensities of 0.30 ± 0.05 and 0.70 ± 0.05, respectively. These values correspond to the mean ± the standard deviation. The shorter lifetime emission is attributed to nonspecifically bound probes residing in the muscle fiber on sites other than SH-1 on the cross-bridge. Previous lifetime studies of S-1 labeled with 1,5-IAEDANS and in solution, where the specificity of the label can be controlled very well, indicate the longer lifetime is from probes bound to SH-1 (Mendelson et al., 1973).

The anisotropy decay data, analyzed by using a sum of exponentials, were best fitted by a sum of two exponentials for both relaxed and rigor fibers in both experimental configurations. These data are summarized in Table I. In view of eq 5, we attribute the shorter relaxation time to motion of the small percentage of nonspecifically bound probes and the longer relaxation time to cross-bridge motion.

A typical anisotropy decay curve for a rigor muscle fiber in the vertical geometry is shown in Figure 2a. This curve shows no decay past the initial decay from the nonspecific probe motion, indicating no detectable cross-bridge motion for times $>7 \times 10^3$ ns.

Figure 2b shows a typical anisotropy decay curve for a relaxed muscle fiber in the vertical geometry, with a relaxation time $\sim 10^3$ ns. As mentioned above, we have applied the theory of time-resolved fluorescence polarization from an ordered assembly (Burghardt, 1985) to these data. In this theory, the steady-state angular distribution and the time-resolved anisotropy decay for subfragment 1 of the myosin molecule in solution are used to construct the time course of the anisotropy decay for the ordered assembly. The free parameters are the diffusion constants D_1, D_2, and D_3 and are determined from the data presented in this paper. When S-1 is considered to be an ellipsoid of revolution with an axial ratio of 3.5, we find $D_1 = D_2 = 1.25 \times 10^5$ s^{-1} and $D_3 = 3 \times 10^5$ s^{-1}.

The time-zero anisotropy is lower for horizontally mounted fibers than for vertically mounted fibers, and the relaxation times are ~ 300 ns for both the rigor and relaxed states (see Table I). The anisotropy decay time for the horizontal rigor fibers is rather uncertain because the time-zero anisotropy for this system is low.

Discussion

When vertically mounted fibers are illuminated with light polarized parallel to the fiber axis, the polarization anisotropy is sensitive only to rotational motions about axes other than the fiber axis. In this configuration, the cross-bridges have a rotational correlation time of 1000 ns. This result is in approximate agreement with a previous study of cross-bridges in unoriented synthetic filaments (Eads et al., 1984). In the vertical geometry, rigor cross-bridges have rotational correlation times of >7000 ns. When the fiber is horizontally mounted so that the excitation light is polarized perpendicular to the fiber axis and the propagation vector has a component along the fiber axis, the polarization anisotropy is sensitive to rotational motions about the fiber axis. In this configuration, it is found that the cross-bridges in both the relaxed and rigor states have rotational correlation times of ~ 300 ns.

We have applied the theory for rotational diffusion in a potential to the data for relaxed fibers. (The theory is not applied to data from rigor fibers, since any model for rigor cross-bridges should include actin dynamics.) For vertically mounted fibers, the best fit to eq 3 and 5 yields rotational diffusion constants of approximately $D_1 = D_2 = 1.24 \times 10^5$ s^{-1} and $D_3 = 3 \times 10^5$ s^{-1}. The exact relationship between the relaxation rates E_j and the remaining free parameters from the diffusion tensor is derived and given in Burghardt (1985). The relationship requires knowledge of the steady-state angular distribution of probes, which has been measured (Wilson & Mendelson, 1983). For horizontally mounted fibers in relaxation, diffusion constants were not recalculated. In this geometry, the angular potential must be described with three instead of two angular dimensions, which complicates the mathematics beyond practicability. However, the theory does indicate [to lowest order approximation—see section e of Burghardt (1985)] that a horizontally mounted fiber should have a correlation time significantly less than that of a vertically mounted fiber. The experimental agreement with this prediction is an argument supporting the validity of the rotational diffusion in an angular potential treatment.

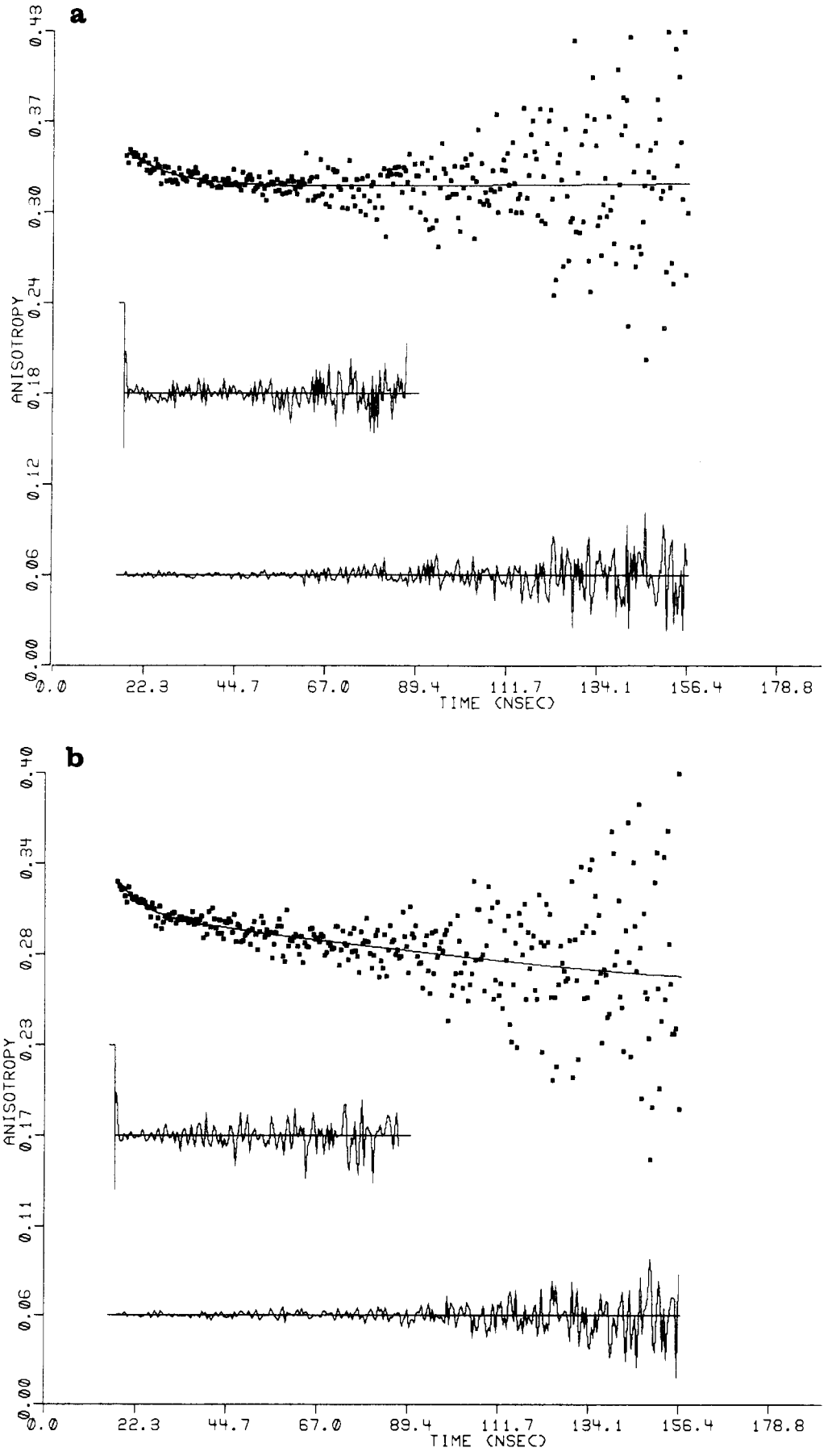

FIGURE 2: Fluorescence anisotropy decay curves. Shown in the top curves are typical fluorescence polarization anisotropy decay curves as a function of time from vertically mounted fibers in (a) rigor and (b) relaxing solution. The middle and bottom curves are the autocorrelation of residuals and the residuals indicating the goodness of the two-exponential fit.

The results indicate that, when dissociated from actin, a cross-bridge in the presence of ATP rapidly investigates many orientations relative to the actin filament. Although the cross-bridge easily encounters the thin filament, it may be necessary that in the encounter the cross-bridge have particular orientation coordinates that change in time if the thick and thin filaments are sliding with respect to each other (i.e., the muscle is contracting). In this case, rapid reorientation of the dissociated cross-bridge is a method of guaranteeing that the cross-bridge will be able to quickly bind to actin in the next step of the cross-bridge cycle.

Rapid cross-bridge rotational motion in a relaxed fiber is consistent with the current understanding of the relaxed state. However, rapid cross-bridge rotational motion in rigor muscle fibers is unusual. This motion is detected by the probes on the cross-bridges in the rigor state only when the fiber is in the horizontal geometry. This observation indicates this anisotropy decay is due to probe rotational motion about the fiber axis. Our data cannot distinguish between probe motion resulting from flexibility in the cross-bridge while attached to the actin filament or from flexibility in the thin filament itself, such that the cross-bridge follows the flexing motions of the thin filament. Flexibility of the actin filament has been reported; however, these motions were several orders of magnitude slower than the 300 ns observed here (Yanagida et al., 1984; Yoshino et al., 1978). Internal motions of other proteins in solution have been observed in the nanosecond time range (Yguerabide et al., 1970; Mendelson et al., 1973).

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