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Rotational Motions of Myosin Heads in Myofibril Studied by Phosphorescence Anisotropy Decay Measurements*

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We studied the rotational Brownian motions of myosin heads, of which the sulfhydryl group was selectively labeled with the triplet probe 5-eosinylmaleimide, in myofibril by using flash-induced phosphorescence anisotropy decay measurements. The anisotropy decay curve under relaxing conditions consisted of a fast (submicrosecond) and a slow (a few microseconds) component and a small constant part as in the synthetic myosin filaments in solution. The decay curves could be analyzed by assuming that a head part, *i.e.* subfragment 1 (S1), wobbles in the first cone and a part connecting S1 and the tail of a myosin molecule of which the length is shorter than subfragment 2 (S2) wobbles in the second cone (a double-cone model); the semiangles of the former and the latter cones were about 30 ° and 50 °, respectively. The rotational freedom of myosin heads was only slightly restricted by the limited space of the filament lattice in myofibrils. Under rigor conditions, no motion of myosin heads was observed in the 10-μs time scale.

Recently, flash-induced absorption anisotropy decay and phosphorescence anisotropy decay measurements (1, 2) were applied to the study of thermal rotational motions of myosin heads by selectively labeling the heads with a triplet probe having a lifetime of a few milliseconds (3, 4). Kinosita *et al.* (4) found that the anisotropy decay for subfragment 1 (S1) was a single exponential over two decades; the analysis showed that the largest diameter of S1 was 14–17 nm if modeled as a prolate ellipsoid of revolution. On the other hand, the anisotropy decay in synthetic myosin filaments consisted of a fast (submicrosecond) and a slow (a few microseconds) component and a small constant part; the fast decay could be explained by a wobbling motion of myosin heads around the head-rod junction within a cone of semiangle 35 ° and the slow decay was attributed to the wobbling motion of a part of the rod portion next to the head, of which length was estimated to be 14 nm, much shorter than that of S2.

In the present study, the above method was applied to an organized system, *i.e.* myofibril. We examined the rotational motions of myosin heads selectively labeled with a triplet probe, 5-eosinylmaleimide, under rigor and relaxing conditions. The effect of spacing of the filament lattice was also examined by changing the osmotic pressure outside the myofibrils.

A preliminary report has been presented previously (5).

MATERIALS AND METHODS

Myofibrils were prepared from rabbit back and leg white muscles (6) and stored in 50% (v/v) glycerol, 0.5 mM $NaHCO_3$, and 5 mM EGTA[1] at −20 °C. The sarcomere length of myofibrils was distributed between 2.2 and 2.8 μm (the average, 2.5 μm). The protein concentration was determined by the biuret reaction and a modified Lowry method as used in the previous work (4; correction was made for the absorbance of labeled dye).

The specific labeling of myosin heads in myofibrils was performed according to the previous work with slight modifications (7, 8). After glycerol was removed by repeated centrifugation at 600 × *g* in the rigor solution (solution A: 60 mM KCl, 5 mM $MgCl_2$, 10 mM Tris maleate buffer (pH 6.8), and 1 mM EGTA), myofibrils (10 mg/ml; the concentration of myosin heads was estimated to be about 20 μM) were incubated in solution A in the presence of 1 mM *N*-ethylmaleimide (NEM; Sigma) for 10 min. It was expected that NEM was bound to thiol (SH) groups on myosin, actin, and so on, except SH1, *i.e.* the most reactive SH group in myosin molecule, of which reactivity was suppressed by the formation of cross-bridges with actin filaments under the rigor condition (9). After free NEM was removed by centrifugation, myofibrils were incubated in solution A containing 20 μM 5-eosinylmaleimide (Molecular Probes) and 10 mM potassium pyrophosphate (PPi). In the presence of MgPPi, a fraction of myosin heads are detached, so that the reactivity of SH1 is expected to recover (7, 8). Three minutes later, the labeling reaction was stopped by adding 1 mM dithiothreitol (DTT). Free 5-eosinylmaleimide, PPi, and DTT were removed by repeated centrifugation in solution A. All procedures were performed at 0 °C in the dark.

We estimated that about 90% of the probes that were incorporated into myofibrils attached to the head part of myosin. The fraction of labeled heads was always around 60%. These values were estimated by measuring the absorbance of the probe 5-eosinylmaleimide at the main peak (extinction coefficient, 8×10^4 M^{-1} cm^{-1}) for labeled myofibrils and myosin extracted from the myofibrils (4) and by assuming that the content of myosin in myofibril is 50% (w/w) (7). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (10) showed that the probe was incorporated into the head part of myosin (Fig. 1, *a–c*), especially the 20,000-dalton fragment containing the SH1 of the head part (Fig. 1, *d* and *e*). The faint line observed below *HC3* in the *right lane* of Fig. 1*e* corresponds to the 70,000-dalton fragment which is subsequently split into 50,000-dalton and 20,000-dalton fragments. The densitometry confirmed the above estimation. Fluorescence micrographs of labeled myofibrils showed that the probes were localized at A-bands, except the central bare zone (Fig. 2).

We measured the ATPase activities (units: micromole of phosphate/(min·mg protein)) of labeled and unlabeled myofibrils and extracted myosin at 25 °C. The Mg-ATPase activity of myofibrils was

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[1] The abbreviations used are: EGTA, [ethylenebis (oxyethylenenitrilo)]tetraacetic acid; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVP, polyvinylpyrrolidone; MOPS, 4-morpholinepropanesulfonic acid.

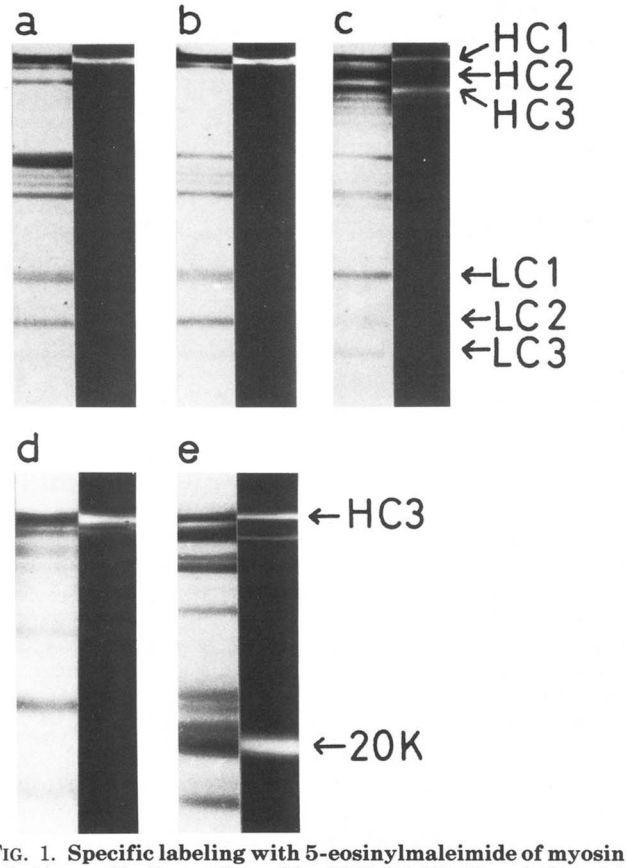

FIG. 1. **Specific labeling with 5-eosinylmaleimide of myosin heads in myofibrils confirmed by SDS-PAGE.** 15 and 5% (w/v) acrylamides were used for a separation gel and a stacking gel, respectively (4). *a*, 5-eosinylmaleimide myofibril; *b*, crude fraction of myosin extracted from 5-eosinylmaleimide myofibril; *c*, crude 5-eosinylmaleimide myosin digested with chymotrypsin; *d*, crude fraction of S1 obtained by chymotryptic digestion of *b*; *e*, tryptic digestion of *d*. In each pair of photographs, the *left lane* shows proteins stained with Coomassie Brilliant Blue R, and the *right lane* shows 5-eosinylmaleimide fluorescence before the staining. In *b*, the extract of 5-eosinylmaleimide myofibril in 0.5 M KCl, 10 mM MOPS (pH 7.0), 5 mM $MgCl_2$, 1 mM EGTA, and 10 mM PPi was examined. In *c*, crude 5-eosinylmaleimide myosin (8 mg/ml) equilibrated with 0.12 M NaCl, 20 mM MOPS (pH 7.0), and 1 mM EDTA was incubated at 25 °C in the presence of 0.05 mg/ml α-chymotrypsin (11). After 10 min, 50 μl of the sample were mixed with 200 μl of a solution containing 1.25% SDS, 1.25% β-mercaptoethanol, and 12.5 mM Tris-HCl buffer (pH 6.8) at 80 °C. The solution was left overnight at room temperature and applied to the gel. In *d*, S1 was obtained by ammonium sulfate fractionation of *c*. In *e*, S1 in 0.5 mM $NaHCO_3$ was incubated with 0.02 mg/ml trypsin at 25 °C for 5 min and then 0.04 mg/ml trypsin inhibitor was added. The sample for SDS-PAGE was prepared as in *c*. Symbols: *HC1, HC2*, and *HC3* correspond to the heavy chains of myosin, rod, and S1, respectively. *LC1, LC2*, and *LC3* show light chains, and *20 K* indicates a 20,000-dalton fragment of S1.

measured in 0.1 M KCl, 5 mM $MgCl_2$, 10 mM Tris maleate buffer (pH 6.8), 4.5 mM ATP and with 0.1 mM $CaCl_2$ (+Ca) or 1 mM EGTA (−Ca). A typical example showed 0.31 (+Ca) and 0.02 (−Ca) for unlabeled myofibrils and 0.17 (+Ca) and 0.09 (−Ca) for labeled myofibrils in which 60% of myosin heads were labeled. This suggests that the actin-activated ATPase activity of labeled heads was modified, although it still existed. (It is known that the rate constants in the kinetic scheme of the myosin ATPase reaction are altered to some extent due to the SH labeling (12).) However, we expect that the mobility of labeled heads will well simulate that of unlabeled ones at least in rigor and relaxing conditions. The K^+-EDTA ATPase activity of extracted myosin was measured in 0.45 M KCl, 50 mM Tris-HCl buffer (pH 7.5), 5 mM ATP, and 1 mM EDTA. A typical example showed 1.76 for unlabeled myosin, 1.44 for myosin extracted from myofibrils treated with 1 mM NEM, and 0.52 for labeled myosin. The extent of the decrease of K^+-EDTA ATPase activity suggested that

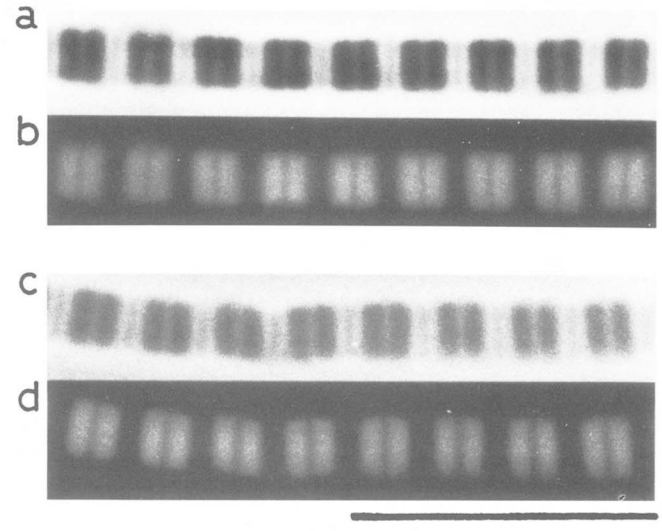

FIG. 2. **Optical micrographs of 5-eosinylmaleimide myofibrils.** *a* and *c*, phase-contrast and *b* and *d*, fluorescence micrographs. Solvent composition: 0.1 M KCl, 5 mM $MgCl_2$, 10 mM Tris maleate buffer (pH 6.8), and 1 mM EGTA (solution B) for *a* and *b* and 53 mM KCl, 2.6 mM $MgCl_2$, 5.3 mM Tris maleate buffer (pH 6.8), 0.5 mM EGTA, 9 mM ATP, and 60% (w/w) sucrose for *c* and *d*. ATP was finally added after myofibrils were well dispersed in the rigor solution containing sucrose. The contrast of the phase-contrast micrograph in *c* was lower than that in *a* because of the high refractive index of the sucrose solution and the swelling of the filament lattice by ATP. *Bar*, 10 μm. ×4,000.

52% (=((1.76 − 0.52)/1.76 − (1.76 − 1.44)/1.76) × 100) of SH1 of myosin heads were labeled. This estimate was consistent with that obtained by the absorbance of the bound probe, *i.e.* 56%. ATP was purchased from Boehringer Mannheim and α-chymotrypsin from Worthington. All chemicals were of reagent grade.

The tension and the stiffness of glycerinated rabbit psoas muscle fibers were measured according to the method described in a previous paper (13). The same method as the above was applied to the labeling of myosin heads in muscle fibers; instead of centrifugation, fibers of which both ends were stuck to a cover glass with double-stick tape were successively immersed in different solutions. The selective labeling of myosin heads was achieved.

Apparatus and measuring procedures for the anisotropy decay measurements were essentially the same as those described in Ref. 4. Phosphorescence anisotropy $r(t)$ was analyzed in terms of the following function by using a least squares program.

$$r(t) = r_0 \sum_{j=1}^{N} \beta_j \exp(-t/\phi_j) \quad \sum_{j=1}^{N} \beta_j = 1 \tag{1}$$

In most cases, the measurements were performed at 0 °C in the presence of 60% (w/w) sucrose to obtain good time resolution. The rotational motions of myosin heads were slowed down by high viscosity at the low temperature. Myofibrils were not disorganized by mixing in such a viscous solution (Fig. 2, *c* and *d*).

RESULTS AND DISCUSSION

Fig. 3 shows that, first, under the rigor condition, the anisotropy does not decay in the 10-μs time scale, indicating that the rotational motion of myosin heads is suppressed because of the binding to thin filaments (14, 15). A small decay was observed at the initial phase in some preparations but disappeared after contraction (post-contraction). Probably, dissociated heads were present at the H zone of myofibrils and bound to thin filaments after contraction. Thus, we conclude that bound heads without ATP have no motion in the 10-μs time scale.

Second, under relaxing conditions we observed a large rapid decay of anisotropy similar to that observed in synthetic myosin filaments in solution (*cf.* Refs. 3 and 4). This indicates

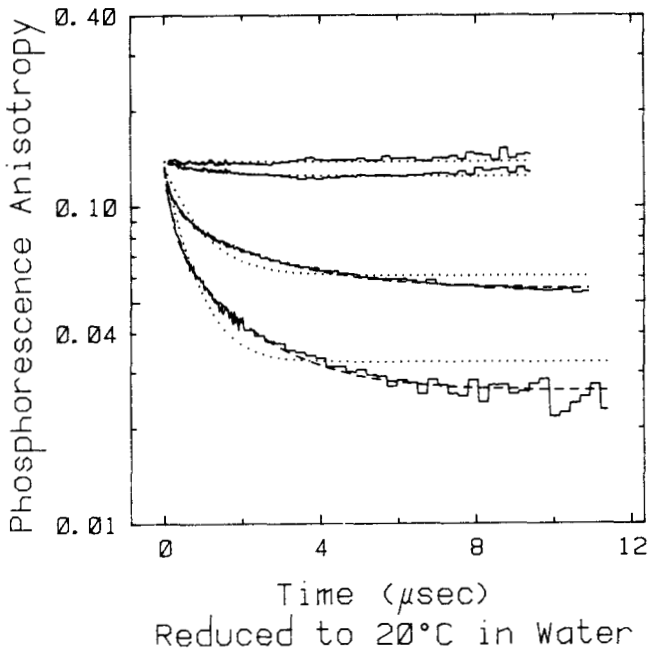

FIG. 3. **Phosphorescence anisotropy decays of 5-eosinyl-maleimide myofibrils under different solvent conditions.** Raw experimental curves (*zigzag lines*), the best-fit curves for Equation 1 with $N = 2$ and $\phi_2 = \infty$ (*dotted lines*) and the best-fit curves for Equation 1 with $N = 3$ and $\phi_3 = \infty$ (*dashed lines*). Myofibrils, 0.7 mg/ml. Conditions: the top *curve* (post-contraction) after 0.1 mM ATP was used up in the rigor solution (solution B in Fig. 2), the concentration of sucrose was increased up to 60% (w/w) by adding supersaturated 75% (w/w) sucrose solution, which was prepared by dissolving 7.5 g of sucrose against 2.5 ml of solution B bathed in a boiling water. Final solvent composition: 53 mM KCl, 2.6 mM $MgCl_2$, 5.3 mM Tris maleate buffer (pH 6.8), 0.5 mM EGTA, 5 mM DTT, and 60% (w/w) sucrose; the second curve (rigor), in the above rigor solution containing sucrose; the third and the fourth curves, in the same rigor solution containing sucrose as the above plus, respectively, 4.5 and 9 mM ATP. The data were obtained at 0 °C in the presence of 60% (w/w) sucrose and the unit of *abscissa* was reduced by a factor of $[(\eta/T)$ at 20 °C in water$]/[(\eta/T)$ at 0 °C in 60% sucrose], where η is the solvent viscosity and T the absolute temperature.

that myosin heads rapidly rotate over a wide range of angle when dissociated from thin filaments. The decay curves were well simulated by Equation 1 containing two exponentials and a constant term. The relaxation times for two exponentials, *i.e.* ϕ_1 and ϕ_2, were, respectively, 0.20 and 1.7 μs for 9 mM ATP, and 0.18 and 2.2 μs for 4.5 mM ATP when (η/T) was reduced to that without sucrose and at 20 °C, where η is the solvent viscosity and T the absolute temperature.

The data could be analyzed by a double-cone model (2, 16) in which a head part taken to be the same as S1 wobbles in the first cone and part of the rod portion next to the head also wobbles in the second cone. The semiangle of each cone, θ_1 and θ_2, and the wobbling rotational diffusion coefficient, D_1 and D_2, in each cone was, respectively, calculated to be 32 ° and 47 °, and 0.40 and 0.09 μs^{-1} for 9 mM ATP. These values are a little smaller than or comparable with those obtained in synthetic myosin filaments in solution (*cf.* Table V in Ref. 4). These results were not influenced by the degree of labeling of myosin heads (20–70% in myofibril; 50–90% in myosin filaments in the previous work (4)), suggesting that the rotational freedom of two heads of myosin is similar to each other.

Incidentally, the constant term of the anisotropy decay curve for 4.5 mM ATP was larger than that for 9 mM ATP (Fig. 3). The constant term is a measure of the rotational freedom of myosin heads. There are at least two factors which contribute to this term, *i.e.* the angular range of the rotation of detached heads and the fraction of attached heads. The concentration effect of ATP observed here will be mainly attributable to the latter factor; a fraction of myosin heads was attached in the presence of 4.5 mM ATP but almost all heads were detached in the presence of 9 mM ATP. In fact, we confirmed that the stiffness (an indicator of the fraction of attached myosin heads) of a muscle bundle in the same solvent containing 60% (w/w) sucrose as used here depended on the concentrations of ATP; the stiffness of a muscle bundle was larger in 4.5 mM ATP than in 9 mM ATP. This effect of ATP was reversible. There would be several factors to keep some fraction of myosin heads attached to thin filaments in 4.5 mM ATP. First, because of the slow diffusion of ATP, the local concentration of ATP around myosin heads may be so low as to induce the formation of a rigor complex. Also, the local environment such as an effective pH and ionic strength around myosin heads may be different from that in the bulk solution, resulting in the increase of the binding affinity of the heads with actin.

In the absence of sucrose, the anisotropy decay in relaxed myofibrils was too fast to be resolved by our instrument. However, the final plateau level of the anisotropy decay could be estimated with reasonable accuracy. The final plateau level (the constant term) of the anisotropy decay in relaxed myofibrils showed a small value comparable to that obtained in synthetic myosin filaments in solution independent of the concentrations of ATP (1–9 mM; *cf.* Table I). It was confirmed that the stiffness of a muscle bundle under these conditions was low, indicating full dissociation of myosin heads. Thus, the concentration effect of ATP described above is peculiar to sucrose. These observations suggest that the angular range of the rotational motion of dissociated heads is as large as that in the synthetic filaments.

Myofibrils are swollen by the addition of ATP as observed under a phase-contrast microscope (*cf.* Fig. 2). Therefore, the large rapid motion of myosin heads observed under the relaxing conditions might be ascribed to the large spacing of the filament lattice induced by the swelling of myofibrils. We examined the effects of lattice spacing on the head motion; the lattice spacing was decreased by increasing the osmotic pressure outside myofibrils on addition of appropriate amounts of PVP (polyvinylpyrrolidone K 30) to the medium. In the relaxing solution (without sucrose) containing 5% (w/v) PVP, the swelling due to ATP of myofibrils was suppressed or myofibrils were rather slightly compressed compared with those under the rigor condition as judged by the phase-contrast microscopy (*cf.* 17, 18). The angular range of the head motion in 5% (w/v) PVP was similar to, or slightly suppressed compared with, that without PVP (*cf.* Table 1). On the other hand, the head motion was largely suppressed

TABLE I

Effects of PVP concentration on the rotational mobility of myosin heads

	PVP concentration (% w/v)				
	Relax				Rigor, 0
	0	5	6	9	
$r(\infty)$[a]	0.020	0.023	0.028	0.078	0.112

[a] Final plateau level of phosphorescence anisotropy, averaged between 600 and 800 μs after the excitation. The lower the level, the larger the average angular range of the head motion. Solvent composition: 0.1 M KCl, 5 mM $MgCl_2$, 10 mM Tris maleate buffer (pH 6.8), 5 mM DTT, 1 mM EGTA, and with (relax; 0, 5, 6, or 9% (w/v) PVP was also added) or without (rigor; no PVP) 4.5 mM ATP. The value of $r(\infty)$ in the relaxing solution without PVP was independent of the ATP concentrations between 1 and 9 mM. Sucrose was not added. Myofibrils, 0.7 mg/ml. Temperature, 0 °C.

but still observed in the presence of 9% (w/v) PVP, where myofibrils collapsed radially and the width became nearly one-half of that without PVP. (Incidentally, the Mg-ATPase activity of myofibrils in the presence of micromolar concentration of Ca^{2+} with 5 or 9% (w/v) PVP was, respectively, about 85 or 50% of that without PVP. It seems that the rotational freedom of the head motion correlates with the actin-activated ATPase activity of myofibrils.) Thus, we conclude that under relaxing conditions, myosin heads in intact myofibrils rotate freely over a wide range of angle, although the wobbling range may be slightly restricted by the limited space of the filament lattice. This result seems to be in contrast to that obtained by Mendelson and his colleagues (19–21); myosin heads have a favored orientation with a relatively small angular range under relaxing conditions. The reason for this contrast might be ascribed to the different methods of preparation, *e.g.* labeling procedure and label itself. Also, it might be attributable to the fact that they estimated the rotational range based on the initial portion of decay curves.

We observed that as ATP was consumed in the absence of Ca^{2+} and sucrose, the final plateau level of the anisotropy decay that had been once decreased to about 0.02 gradually increased up to the level close to that of the initial rigor state; thus, the reversibility of the effect of ATP was confirmed. Another important point of this observation is that 5-eosinylmaleimide-labeled myosin heads could still bind to thin filaments after the repeated irradiation of laser light.

The recovery process to the rigor state accompanying the consumption of ATP could not be examined in the presence of 60% (w/w) sucrose because the ATPase activity of myofibrils was too low in such a high concentration of sucrose even in the presence of Ca^{2+}. The reason is not clear (22). However, we confirmed that by using muscle fibers (both labeled and unlabeled) the rigor tension was developed and the stiffness was increased by changing the solution containing sucrose from the relax to the rigor, indicating that the reversible association and dissociation of cross-bridges occur in the presence of 60% (w/w) sucrose.

The present results clearly showed that myosin heads in myofibrils extensively rotate and translate (due to the rotational motion of the rod portion) under relaxing conditions and are immobilized under rigor conditions. The rotational freedom of myosin heads was not largely disturbed by the limited space of the filament lattice.

It should be noted here that the thermal rotational motions of the heads observed here are not necessarily the same as the "active" motions assumed in the conventional theory of muscle contraction (23, 24). It has not yet been possible to detect the active motions of myosin heads by use of the present method. Myofibrils may not be suited for this purpose because the lattice structure is disorganized by contraction. In order to get information not only on the orientation of myosin heads to the long axis of myofibril but also on the rotational motions of the heads during tension development, we need to apply the present method to the more organized and oriented system, *i.e.* muscle fibers (*cf.* 20, 25–28).

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