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 myofibril.

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 NaHCO₃, and 5 mM EGTA 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 modification (7, 8). After glycerol was removed by repeated centrifugation at 600 × g in the rigor solution (solution A: 60 mM KCl, 5 mM MgCl₂, 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 MgF₄, 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 × 10⁴ 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 60% (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. 1c 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, ethyleneglycol (oxymethylene)tritol; tetrazetcic acid; NEM, N-ethylmaleimide; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVP, polyvinylpyrrolidone; MOPS, 4-morpholinepropanesulfonic acid.
measured in 0.1 M KCl, 5 mM MgCl₂, 10 mM Tris maleate buffer (pH 6.8), 4.5 mM ATP and with 0.1 mM CaCl₂ (+Ca) or 1 mM EGTA (−Ca). A typical example showed 0.51 (+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 indicates 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

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...
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, $\tau_1$ and $\tau_2$, 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 $\eta/T$ was reduced to that without sucrose and at 20 °C, where $\eta$ 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 $S_1$ wobbles in the second cone. The semiangle of each cone, $\theta_1$ and $\theta_2$, 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 detached 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 inside myofibrils and the wobbling motion of detached heads was also suppressed (Table I). Therefore, the large rapid motion of myosin heads observed under the relaxing conditions might be explained by the large spacing of the filament lattice induced by the swelling of myofibrils. 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 I). On the other hand, the head motion was largely suppressed in the relaxing solution (without sucrose) containing 5% (w/v) PVP, the swelling due to ATP of myofibrils was suppressed; 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 I).

<p>| TABLE I |
| Effects of PVP concentration on the rotational mobility of myosin heads |</p>
<table>
<thead>
<tr>
<th>PVP concentration (% w/v)</th>
<th>Relax</th>
<th>Rigor</th>
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</thead>
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<tr>
<td>0</td>
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<td>0.023</td>
</tr>
<tr>
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<td>0.078</td>
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<tr>
<td>6</td>
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$^a$ 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-ethyl-

nylmaleimide-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. 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 (cf.

REFERENCES