Exchange of the Fluorescence-Labeled 20 000-Dalton Light Chain of Smooth Muscle Myosin†

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ABSTRACT: The 20 000-dalton light chain of smooth muscle myosin was exchanged with exogenous light chain in a solution containing 0.5 M NaCl and 10 mM EDTA at 40 °C. The light chain was almost completely exchanged within 30 min under the above conditions. The exchange was markedly inhibited either below 37 °C or in the presence of Mg^{2+} concentrations higher than 10 μM. The 20 000-dalton light chain was selectively labeled of a single thiol (Cys-108) with 5-[[2-[(iodoacetyl)amino]ethyl]amino-naphthalene-1-sulfonic acid (1,5-IAEDANS). The labeled light chain was exchanged stoichiometrically into myosin and was used as a probe to investigate the conformation of smooth muscle myosin. The resulting myosin hybrids showed enzymatic properties virtually identical with those of the control, untreated myosin; i.e., actin-activated ATPase activity was dependent on the 20 000-dalton light-chain phosphorylation catalyzed by myosin light chain kinase, and the 10S–6S conformational transition of myosin correlating with the changes in ATPase was also affected either by the light-chain phosphorylation or by the change in the ionic strength. Steady-state fluorescence antisotropy measurements were performed by varying the temperature. The Perrin–Weber plots were constructed in order to obtain information about the average rotational mobility of the probe and to estimate the rotational correlation time for the AEDANS–myosin head. The fluorescence probe on the 20 000-dalton light chain was found to be quite immobile as indicated by its limiting anisotropy (A_0 = 0.33). The rotational correlation time of attached AEDANS at 20 °C increased from 8.7 ns in AEDANS–20 000-dalton light chain to 16.8 ns when incorporated into myosin subfragment 1 (S-1), but this value was significantly smaller than that calculated for an anhydrous, rigid sphere of the size of S-1, suggesting that the light-chain binding site of S-1 is partially flexible and/or the light chain itself has considerable motional freedom with respective to the rest of the S-1 structure. The binding of AEDANS-labeled light chain to 6S myosin resulted in only a slight increase in the rotational correlation time over that of S-1 while the rotational correlation time of AEDANS-labeled light chain bound to 10S myosin was significantly larger than those obtained with 6S myosin. Analogously, the rotational correlation time of dephosphorylated H-meromyosin was significantly larger at low ionic strength (0.03 M KCl) than that at high ionic strength (0.4 M KCl). These results suggest that the conformational transition of smooth muscle myosin from 6 S to 10 S brings about a change in conformation of the 20 000-dalton light-chain binding site of myosin which is located in the head–rod junction of myosin.

It is known that phosphorylation of smooth muscle myosin is an integral component of the regulatory mechanism of smooth muscle (Sobieszek, 1977; Aksoy et al., 1976; Chacko et al., 1977; Ikebe et al., 1977; Sherry et al., 1978). Phosphorylation of serine-19 on the 20 000-dalton light chain of myosin by myosin light chain kinase increases the actin-activated ATPase activity of myosin, and it is thought that this causes the initial development of tension in muscle cells. The bulk of evidence has been accumulated for the phosphorylation hypothesis for the activation of smooth muscle (Hartshorne, 1987), and it was shown recently that the phosphorylation of myosin by myosin light chain kinase is necessary and sufficient for the initiation of contraction using the microinjection of the constitutively active myosin light chain kinase into the isolated intact single smooth muscle cell (Itoh et al., 1989). However, it is still obscure how the phosphorylation of the 20 000-dalton light chain affects the ATPase site to activate ATP hydrolysis. Although the details of the molecular mechanism of communication between the regulatory site and the catalytic site are not well understood, some progress has been made in the molecular change induced by phosphorylation. It was found several years ago that monomeric smooth muscle myosin can form two distinct conformations, i.e., the folded (10 S) and extended (6 S) (Suzuki et al., 1982; Trybus et al., 1982; Onishi & Wakabayashi, 1982; Craig et al., 1983). Moreover, these two conformations were characterized by distinct enzymatic properties (Ikebe et al., 1983), and it was also found that the phosphorylation of 20 000-dalton light chain affects the 10S–6S transition (Trybus et al., 1982; Trybus & Lowey, 1984; Craig et al., 1983; Ikebe et al., 1983; Onishi et al., 1983). These results had led to the shape–activity hypothesis in which the same component of the 10S–6S transition is involved in the regulation of ATPase activity (Ikebe et al., 1983, 1984).

We have attempted to determine which components of the transition might determine the enzymatic activation of smooth muscle myosin, and it was suggested that the change in the conformation at the subfragment 1/subfragment 2 (S-1/S-2)[1]

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[1] Abbreviations: S-1, myosin subfragment 1; S-2, myosin subfragment 2; HMM, heavy meromyosin; 1,5-IAEDANS, 5-[[2-[(iodoacetyl)-amino]ethyl]amino]naphthalene-1-sulfonic acid; DTT, dithiothreitol; MLCK, myosin light chain kinase; HPLC, high-performance liquid chromatography; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate.

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junction is the determinant for the regulation of ATPase activity based on the following evidence. (1) The 10S myosin is more resistant to proteolysis at the S-1/S-2 junction than 6S myosin (Onishi & Watanabe, 1984; Ikebe & Hartshorne, 1984). This resistance to proteolysis was also found in dephosphorylated HMM at the S-1/S-2 junction, which does not form the folded structure because of the lack of the tail of the myosin molecule (Ikebe & Hartshorne, 1984). (2) The actin-activated Mg^{2+}-ATPase activity of S-1 which retains an intact 20000-dalton light chain is not regulated by phosphorylation while the ATPase activity of acto–HMM is regulated by phosphorylation (Ikebe & Hartshorne, 1985a). (3) The antibody which recognizes the 17000-dalton light chain and inhibits the formation of 10S myosin activates the actin-activated Mg^{2+}-ATPase of both dephosphorylated myosin and also HMM (Higashihara et al., 1989). The antibodies which recognized S-2 inhibit the depression of myosin Mg^{2+}-ATPase at low ionic strength (Ito et al., 1989; Higashihara & Ikebe, 1990). However, it is not clear how the change in the conformation at the S-1/S-2 junction affects the ATP hydrolysis at the active site. In this study, we used the fluorescence-labeled 20000-dalton light chain as a probe to investigate the conformation of smooth muscle myosin.

Materials and Methods

Proteins were prepared by using the following methods: smooth muscle myosin from frozen turkey gizzards (Ikebe & Hartshorne, 1985b), myosin light chain kinase from frozen turkey gizzards (Ikebe et al., 1987), myosin light chain phosphatase (Pato & Kerc, 1985) from frozen turkey gizzards, calmodulin from bull testes (Walsh et al., 1983), myosin light chains (Ikebe et al., 1988) and actin from rabbit skeletal muscle (Driska & Hartshorne, 1975). Myosin subfragment 1 (S-1) and heavy meromyosin (HMM) were prepared from myosin by proteolysis with *Staphylococcus aureus* protease (SAP) (Pierce Chemical Co.) as described previously (Ikebe & Hartshorne, 1985a). ATPase activity was measured at 25 °C as described previously (Ikebe & Hartshorne, 1985a). Assay conditions are given in the figure legends.

5-[[2-[(Iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid (1,5-IAEDANS) was obtained from Molecular Probes, Inc. Cys-108 of the 20000-dalton light chain of myosin was labeled with 1,5-IAEDANS by a modified method of Takashi et al. (1976). The 20000-dalton light chain (2–5 mg/mL) was incubated at 25 °C for 90 min in the presence of 0.03 M KCl, 30 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 5 mM DTT to reduce disulfides. After DTT and EDTA were removed by Sephadex G-50 gel filtration (Penefsky, 1977), the light chain was incubated at 0 °C in the presence of 2 mM 1,5-IAEDANS for 20 min. Unreacted 1,5-IAEDANS was removed by Sephadex G-50 gel filtration. The stoichiometry of the modification was estimated by titrating the free sulfhydryl residues of the light chain with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) by the method of Ellman (1959). One mole of SH per mole of light chain was labeled with IAEDANS. AEDANS-labeled or unlabeled 20000-dalton light chain was phosphorylated in 0.3 M KCl, 30 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 20 μg/mL MLCK, 5 μg/mL calmodulin, and 1 mM [γ-^{32}P]ATP or cold ATP at 25 °C for 30 min. Phosphorylation of the light chain was determined by measuring the incorporated ^{32}P according to the method of Walsh et al. (1983) as well as by urea gel electrophoresis (Perrie & Perry, 1970). The light chains were completely phosphorylated under these conditions (1 mol of P/mol of light chain). The 20000-dalton light chain of myosin (0.5–2 mg/mL) or S-1 (0.26–1.04 mg/mL) was exchanged with 10 molar excess AEDANS-labeled light chain in 0.5 M NaCl, 30 mM Tris-HCl, pH 7.5, 10 mM EDTA, 10 mM DTT, and 10 mM ATP at 40 °C for 30 min. The light-chain-exchanged myosin or S-1 was purified by a TSK G 4000 SW gel filtration column (300 × 7.5 mm) and a TSK-DEAE 5PW column (75 × 7.5 mm), respectively, attached with a Perkin-Elmer Series 4 HPLC system. The amount of exchanged light chain was estimated by counting the radioactivity of phosphorylated light chain. The concentration of myosin or S-1 did not affect the extent of exchange of the light chain within the tested concentration range. The Mg-ATPase activity was not altered by incubation under exchange conditions, both in the presence and in the absence of exogenous light chain.

Fluorescence anisotropy of the labeled myosin was measured with an SLM 8000 spectrofluorometer equipped with a set of Glan–Thompson prism polarizers in T format (Takashi & Kasprzak, 1987) or with an SLM SpF-500C spectrofluorometer with a polarization accessory. Fluorescence anisotropy was calculated according to the equation: $A = (I_\parallel - I_\perp)/(I_\parallel + 2I_\perp)$, where $I_\parallel$ and $I_\perp$ represent the emission intensities with the analyzers vertically and horizontally oriented, respectively. The fluorescence was observed at 467 nm using an excitation wavelength of either 360 or 390 nm. In the present work, the ratio of T/η was varied by changing the temperature of the solution by means of a constant-temperature water bath circulating through the cell holder of the fluorometer, and corrections were made for the temperature dependence of the viscosity where appropriate. Addition of sucrose at concentrations higher than 20% to solutions of AEDANS conjugated to 10S myosin led to decreases in anisotropy rather than the increase expected if sucrose only changed viscosity. Therefore, anisotropy measurements at constant temperature of the viscosity dependence were not performed for the present study. The anisotropy data were analyzed by a Perrin–Weber plot which is based on the equation (Perrin, 1934; Weber, 1952):

$$1/A = (1/A_0)(1 + \tau kT/V_\eta) = (1/A_0)(1 + \tau/\phi) \quad (1)$$

where A is the anisotropy at the absolute temperature T, A_0 is the limiting anisotropy at $T/\eta \rightarrow 0$, τ is the fluorescence lifetime, ϕ is the rotational correlation time, k is the Boltzmann constant, V is the molecular volume, and η is the viscosity of the solution. In the case where the Perrin–Weber plot was significantly curved, the rotational correlational time was calculated from eq 1 by using a value of A_0 derived from extrapolation of a straight portion of a plot of $1/A$ vs T/η.

Fluorescence lifetimes were measured and analyzed as sums of exponentials as described in a previous report (Takashi & Kasprzak, 1987). The mean lifetime of the decay, defined below, was used to calculate the rotational correlation time: $\langle\tau\rangle = \sum\alpha_i\tau_i^2/\sum\alpha_i\tau_i$, where α_i is the preexponential term and τ_i is the fluorescence decay constant.

Gel electrophoresis was carried out on a 10–25% polyacrylamide gradient slab gel using the discontinuous buffer system of Laemmli (1970). Molecular weights were estimated by using the following standards: myosin heavy chain (205000), β-galactosidase (116000), phosphorylase *b* (97000), bovine serum albumin (66000), ovalbumin (45000), carbonic anhydrase (29000), smooth muscle myosin light chains (20000 and 17000), and lysozyme (14300). The protein concentrations were estimated by spectrophotometric measurements for calmodulin (A_{277} = 1.9) (Watterson et al., 1976), myosin (A_{277} = 4.54), and 20000-dalton light chain (A_{277} = 3.37) (Hathaway & Haeberle, 1983) or by the method of Bradford (1976) or the biuret reaction (Itzhaki & Gill, 1964).

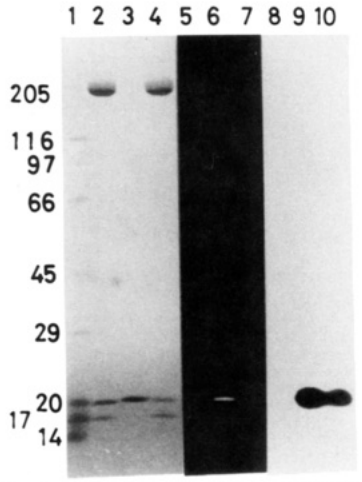

FIGURE 1: SDS gel electrophoresis of the 20000-dalton light chain and light-chain-exchanged myosin. Lane 1 is molecular weight standards. Lanes 2, 5, and 8 are control unexchanged myosin, lanes 3, 6, and 9 are AEDANS-labeled and phosphorylated 20000-dalton light chain, and lanes 4, 7, and 10 are myosin exchanged with phosphorylated and AEDANS-labeled 20000-dalton light chain. The gel was stained by Coomassie Brilliant Blue R-250 (Sigma) (lanes 1–4) or illuminated with ultraviolet rays (lanes 5–7). Lanes 8–10 show autoradiography; 10 μg of unexchanged myosin, 1 μg of light chain, and 10 μg of exchanged myosin were applied to the gel.

RESULTS

The 20000-dalton light chain of myosin was exchanged with phosphorylated AEDANS-labeled 20000-dalton light chain, and the light-chain-exchanged myosin was purified with gel filtration as described under Materials and Methods. As shown in Figure 1, AEDANS-labeled phosphorylated light chain was incorporated into myosin. In typical experiments, approximately 80% of the labeled foreign myosin light chain was exchanged into myosin judged from the incorporated radioactivity of myosin. Dephosphorylated AEDANS-labeled light chain was similarly exchanged and incorporated into myosin (data not shown). The light-chain exchange was also carried out in the absence of ATP, and virtually the same extent of exchange was observed (data not shown). The effects of KCl and NaCl on exchange were also tested. Between 0.15 and 0.5 M, both NaCl and KCl provided a good extent of exchange of the light chains. The presence of EDTA was essential to achieve good light-chain exchange, and in the presence of more than 10 μM $MgCl_2$, little exchange of the light chain was observed. The effect of temperature on light-chain exchange was also studied. Below 37 °C, the extent of exchange was significantly reduced (18% at 37 °C for 30 min), and efficient exchange was only obtained above 40 °C. The maximum extent of exchange was obtained when the incubation was carried out more than 30 min. It should also be mentioned that although the 20000-dalton light chain of myosin was exchanged with the exogenous 20000-dalton light chain, under the conditions described above, washing of myosin at 40 °C in the presence of 10 mM EDTA did not remove the light chain.

Enzymatic properties of the light-chain-exchanged myosin were examined. First, the KCl concentration dependence of Mg^{2+}-ATPase activity of the myosin exchanged with the dephosphorylated AEDANS-labeled light chain was determined. The ATPase activity of the light-chain-exchanged myosin [4.5 nmol/(min·mg) at 0.5 M KCl] decreased with lowering KCl concentration and reached 0.5 nmol/(min·mg) at 0.15 M KCl as was observed for the nonexchanged myosin (data not shown). It was shown previously that the marked decrease in the ATPase activity is due to the formation of the 10S conformation (Ikebe et al., 1983); therefore, this suggests that the light-chain-exchanged myosin also forms the 10S conformation at low ionic strength. The conformation of myosin whose light chain was exchanged with the AEDANS-labeled light chain was monitored by using gel filtration. It was shown previously (Trybus et al., 1982; Ikebe et al., 1983) that 10S and 6S myosins elute at different positions in gel filtration columns according to their difference in the shape of the molecule. The myosin exchanged with the AEDANS-labeled light chain eluted at the same position as 10S myosin at 0.085 M KCl and 6S myosin at 0.5 M KCl, respectively (data not shown). The effect of light-chain phosphorylation on the ATPase activity of exchanged myosin was also examined. Figure 2 shows the KCl concentration dependence of the Ca^{2+}-ATPase activity of myosin exchanged with AEDANS-labeled light chain. Ca^{2+}-ATPase activity of the dephosphorylated myosin decreased significantly below 0.3 M KCl, and it is known that this is due to the change in myosin conformation from 6 S to 10 S (Ikebe et al., 1983). On the other hand, when the exchanged light chain was phosphorylated, the depression of the ATPase activity was abolished. This is similar to the unexchanged myosin (Ikebe et al., 1983), and it is known that the change in the KCl concentration dependence of ATPase activity is due to inhibition of 10S myosin formation by phosphorylation.

The myosin exchanged with AEDANS-labeled light chain (AEDANS–myosin) was phosphorylated (approximately 2 mol of P/mol of light chain), and the actin-activated ATPase

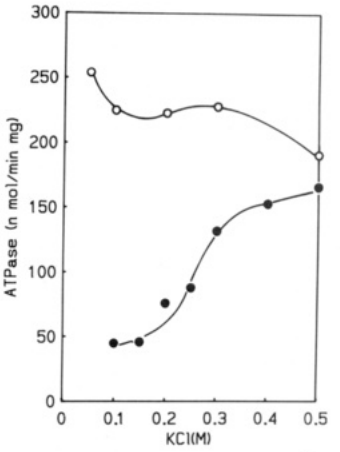

FIGURE 2: KCl concentration dependence of Ca^{2+}-ATPase activity of myosin exchanged with AEDANS-labeled 20000-dalton light chain. Ca^{2+}-ATPase activity of myosin exchanged with phosphorylated AEDANS-labeled 20000-dalton light chain was measured in 30 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, and 0.5 mM ATP before (○) or after (●) incubation with 1 mM EGTA and myosin light chain phosphatase, in a solution containing 30 mM Tris-HCl, pH 7.5, and 1 mM EGTA at 25 °C for 15 min.

Table I: Actin-Activated ATPase Activity of AEDANS–20-kDa Light-Chain Myosin[a]

		act. (nmol of P min^{-1} mg^{-1})	
		without F-actin	with F-actin
exchanged	1 mM $MgCl_2$	0.8	18.0
	10 mM $MgCl_2$	6.5	55.5
unexchanged	1 mM $MgCl_2$	0.8	14.5
	10 mM $MgCl_2$	6.0	54.1

[a] The 20000-dalton light-chain-exchanged myosin was phosphorylated at 25 °C for 20 min in the presence of 0.085 M KCl, 30 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 μg/mL MLCK, 5 μg/mL calmodulin, and 0.2 mM ATP. Mg^{2+}-ATPase activity was measured in 0.085 M KCl, 30 mM Tris-HCl, pH 7.5, and 1 mM (or 10 mM) $MgCl_2$ in the presence and absence of 1.0 mg/mL F-actin.

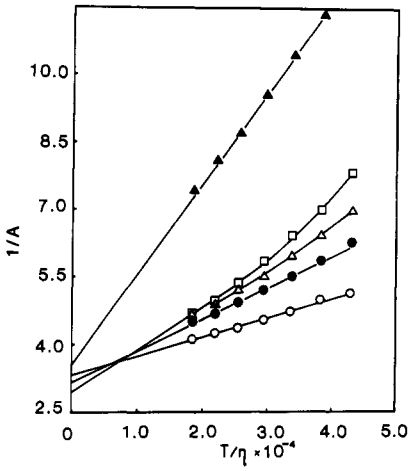

FIGURE 3: Perrin–Weber plots of AEDANS-labeled light-chain-exchanged smooth muscle myosin and subfragment 1. Fluorescence of AEDANS-labeled 20 000-dalton light chain [5 μM (▲)] and AEDANS-labeled 20 000-dalton light-chain-exchanged dephosphorylated myosin [1.16 μM (○, △)], phosphorylated myosin [1.16 μM (●)], and subfragment 1 [3.2 μM (□)] was measured in 30 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, and 0.15 M (○, ●, ▲, □) or 0.5 M KCl (△).

activity was measured. As shown in Table I, Mg^{2+}-ATPase activity of AEDANS–myosin was markedly activated by actin when the 20 000-dalton light chain was phosphorylated, and the activity was similar to the unexchanged myosin. The foregoing results show that myosin in which the 20 000-dalton light chain is exchanged with AEDANS-labeled 20 000-dalton light chain has similar enzymatic and conformational properties to those of native unexchanged myosin.

Fluorescence anisotropy measurements can reveal the presence of segments of proteins that are flexible. In the present work, we have used AEDANS conjugated to the 20 000-dalton light chain associated with myosin heavy chain as a probe of molecular flexibility and measured steady-state fluorescence anisotropy to estimate the rotational correlation times for the AEDANS–myosin head. Figure 3 shows the Perin–Weber plot of fluorescence anisotropy. The data verify that the linear fit to the Perrin–Weber equation is valid for the AEDANS–light chain and the AEDANS–10S myosin conjugates at temperatures from 5 to 35 °C and show that the limiting anisotropy of AEDANS attached to Cys-108 of the light chain (A_0 = 0.331 at λ_{ex} = 390 nm) is very similar to the fundamental anisotropy (A_f = 0.352 at λ_{ex} = 390 nm) (Hudson & Weber, 1973), indicating that the bound dye undergoes little local, depolarizing motion in the binding site. However, as seen in Figure 3, an upward curvature at high values of T/η was observed in the plot for solutions of the AEDANS–S-1 and –6S myosin conjugates. This departure could arise from partial dissociation of a segment in the vicinity of the cystein residue of the 20 000-dalton light chain from myosin heavy chain under the conditions employed, and this temperature effect on the changes in anisotropy was found to be reversible. Using the values obtained for the lifetime (see Materials and Methods) and the A and A_0 values from Figure 3, we have calculated the rotational correlation time. The obtained results are summarized in Table II. The rotational correlation time of 8.7 ns at 20 °C for AEDANS bound to the 20 000-dalton light chain was obtained, and this value remained unchanged upon phosphorylation within an experimental error of 2% (data not shown). The rotational correlation time expected for the light chain if it were a rigid sphere with a hydration of 0.20~0.28 g of H_2O/g of protein has a value of 7.60–8.33 ns. Thus, the slightly larger value for the

Table II: Rotational Correlation Times of AEDANS–Smooth Muscle Myosin

protein	mol wt (×10⁻³)	[KCl] (M)	φ (ns)[a]
LC_{20}	20	0.15	8.7 ± 0.5
		0.5	8.7 ± 0.5
S-1	130	0.15	15.6 ± 1.3[c]
		0.5	16.8 ± 1.9[c]
DP-myosin	480	0.15	37.8 ± 1.0
		0.5	20.0 ± 1.7
P-myosin	480	0.15	22.2 ± 0.8
		0.5	20.1 ± 1.3
S-1 (skeletal)[b]	112	0.3	75.7 ± 4

[a] φ values at 20 °C were calculated by using the Perrin equation (eq 1) where φ = 15.2, 13.8, and 14.3 ns for AEDANS–20 000-dalton light chain, 10S myosin, and 6S myosin, respectively, and are given with standard deviations. [b] Myosin S-1 (A1) (MW = 112 000; Magossian et al., 1981) from rabbit skeletal muscle is labeled with AEDANS at Cys-707. φ values were calculated by employing φ = 20 ns for AEDANS–S-1 (Mendelson et al., 1973; Tao & Lamkin, 1981). [c] φ values were calculated by assuming τ = 15.0 ns for S-1(AEDANS–LC). DP, dephosphorylated; P, phosphorylated.

rotational correlation time which is observed experimentally indicates that the light chain is rigid, that the fluorescence probe is firmly attached, and that the light chain has an asymmetric shape as reported earlier on the regulatory light chains from a variety of sources (Stafford & Szent-Gyorgyi, 1978). When the labeled light chain was incorporated into S-1, the rotational correlation time increased to 16.8 ns. However, this value of the rotational time for AEDANS–S-1 is much smaller than those previously reported for anisotropy decay measurements (94–141 ns at 20 °C) (Mendelson et al., 1973; Botts et al., 1982) and that obtained in the present study from the steady-state anisotropy measurements (75.7 ns) of AEDANS rigidly attached to Cys-707 of the S-1 heavy chain (Table II). Furthermore, it is considerably faster than that calculated for an anhydrous, rigid sphere of the size of smooth muscle myosin S-1 (φ=39 ns). These results suggest that a region of the light-chain segment to which the probe is attached binds in part to a flexible segment of the S-1 heavy chain and/or that a region near or around Cys-108 of the light-chain segment to which the probe is bound has considerable freedom of motion relative to the rest of the S-1 structure. Moreover, when the labeled light chain was incorporated into myosin at high ionic strength where smooth muscle myosin forms the 6S conformation, the rotational correlation time increased, but it was only slightly larger than that of S-1 (Table II), suggesting that the heavy-chain segment in the S-1 portion of myosin where the labeled light chain is bound still retains considerable segmental flexibility and/or that a region of the light-chain segment in myosin to which the label is attached has motional freedom independent of the rest of the myosin molecule. On the other hand, when dephosphorylated myosin underwent the 6S–10S transition by varying KCl concentrations from 0.5 to 0.05 M, this transition was accompanied by a considerable increase of the rotational correlation time from 20.0 to 37.8 ns, suggesting changes in segmental mobility in the vicinity of the chromophore binding site of the light chain. Moreover, it was found that upon phosphorylation the rotational correlation time of myosin at 0.15 M KCl decreased to the value similar to that observed at high ionic strength (6S myosin). This is consistent with the fact that the phosphorylation favors the 6S conformation even at low ionic strength (Trybus et al., 1982; Ikebe et al., 1983; Onishi et al., 1983; Craig et al., 1983).

The observation that 10S myosin exhibits an increased rotational time raised the question of what is inducing the conformational change that is likely to be taking place in the

Table III: Steady-State Anisotropy Parameters for AEDANS-Labeled HMM[a]

expt	[KCl] (M)	A_0^{-1}	A^{-1} [b]	ϕ (ns)[c]
DP-HMM	0.03	3.25	5.42	22.4
	0.4	3.10	5.82	17.0
P-HMM	0.03	3.05	5.63	17.7
	0.4	3.10	5.87	16.7

[a] The steady-state fluorescence anisotropy of AEDANS–HMM (1.2–1.3 μM) was measured in a solution containing 30 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, and 0.03 or 0.4 M KCl. The average uncertainty for the measurements was less than 0.004 anisotropy unit. [b] T = 20 °C and λ_{ex} = 360 nm. [c] Calculated by using eq 1 where τ = 15.0 ns. DP, dephosphorylated; P, phosphorylated.

head–rod junction of the myosin molecule. This conformational change could originate from (a) the influence of the folded tail of the myosin interacting with the head–rod junction and/or (b) the changes in the vicinity of the neck–rod junction itself that may be associated with the regulation.

To clarify the above point, further studies were undertaken with smooth muscle HMM, which is regulated by phosphorylation but unable to form the folded structure (10S conformation) because of lack of the myosin tail.

The labeled light chain was exchanged into HMM, and the steady-state fluorescence anisotropy for AEDANS bound to dephosphorylated and phosphorylated HMM in solution was measured as a function of temperature at 0.03 and 0.4 M KCl and plotted according to eq 1, and subsequently the rotational correlation time was yielded as shown in Table III. The rotational correlation time for dephosphorylated HMM was dependent on ionic strength, and the value was found to be larger at lower ionic strength as was observed with myosin, though to a lesser extent than for dephosphorylated myosin (Tables II and III). Phosphorylation of labeled HMM at 0.03 M KCl decreases the rotational correlation time from 22.4 to 17.7 ns, and its dependence on ionic strength was significantly less than that of dephosphorylated HMM.

The results are consistent with the notion that dephosphorylated HMM does undergo a localized conformational change in the region of the head–rod junction in a [KCl]-dependent manner (Ikebe & Hartshorne, 1984, 1985a; Harthsorne & Ikebe, 1987; Suzuki et al., 1985). Furthermore, the present results indicate that a change in the segmental mobility in the head–rod junction as indicated by its altered rotational correlation time is accompanied by the 6S–10S transition and that the conformational change occurs independent of the interaction of the myosin tail with the head–rod junction. It is also suggested on the basis of the present results that both events a and b described above are likely to contribute to a change in the rotational correlation time that was observed with myosin accompanied by the 6S–10S transition but only the latter may have relevance to the regulation.

Discussion

In the present study, we succeeded in exchanging the exogenous smooth muscle 20 000-dalton light chain with the intrinsic light chain of smooth muscle myosin. The essential conditions for the exchange are different from those for the exchange of skeletal muscle myosin DTNB light chain by the smooth muscle 20 000-dalton light chain or exogenous skeletal muscle DTNB light chain (Morita et al., 1989). In the case of the exchange of the regulatory light chain on skeletal muscle myosin, the presence of ATP is required to protect the myosin from thermal denaturation at 37 °C, whereas the thermal denaturation of smooth muscle myosin does not occur even in the absence of ATP at 40 °C. On the other hand, the removal of divalent cation was essential to achieve the 20 000-dalton light-chain exchange. It is known that the regulatory light chain of scallop myosin is removed by washing with EDTA (Chantler & Szent-Gyorgyi, 1980). It was shown previously for skeletal muscle myosin (Wikmann-Coffelt, 1979) that the removal of divalent cation is essential for DTNB light-chain exchange. This suggests that the binding of divalent cation on the regulatory light chain strengthens the association of the regulatory light chain and heavy chain both for skeletal and for smooth muscle myosins. In contrast to scallop myosin, EDTA washing did not remove 20 000-dalton light chain from smooth muscle myosin. This suggests that the dissociation constant of the regulatory light-chain binding of smooth muscle myosin is much lower than that of scallop myosin. This is consistent with the finding that the good extent of exchange required incubation more than 30 min, which suggests the dissociation rate of light chain is quite slow. Recently, Trybus and Lowey (1988) reported that the 20 000-dalton light chain can be removed from myosin by using an anti-20 000-dalton light-chain antibody column. It was reported that the light-chain-deficient myosin has a tendency to aggregate and does not form a proper folded conformation. Re-addition of the light chain, however, restored the solubility of myosin. Our results are consistent with their results that the 20 000-dalton light-chain-exchanged myosin formed a folded conformation at low ionic strength. Furthermore, the light-chain-exchanged myosin (both native 20 000-dalton light chain and AEDANS-modified light chain) retained virtually the same several properties as native myosin, i.e., [KCl] dependence of Mg^{2+}-ATPase activity, 10S–6S conformational transition, and phosphorylation-dependent actin-activated Mg^{2+}-ATPase activity. Therefore, it may be concluded that the light-chain-exchanged myosin has the same biological activities and properties as native myosin.

In contrast to skeletal muscle myosin, SH-1 and SH-2 which are located in the C-terminal domain of smooth muscle S-1 are not highly reactive (Yamaguchi et al., 1970; Cohen et al., 1976), and modification of smooth muscle myosin with sulfhydryl reagent initially produces labeling of three cysteine residues of the 17 000-dalton light chain. In addition, the modification of SH-1 alters the properties of myosin (Chandra et al., 1985). Using the exchange of light chain, we introduced the fluorescence probe specifically into the myosin molecule without altering the functional properties. Gizzard 20 000-dalton light chain contains only one cysteine residue (Cys-108), and this was modified with IAEDANS to introduce the site-specific fluorescence probe.

We measured the fluorescence anisotropy of chemically labeled myosin in which AEDANS was selectively attached at Cys-108 of the 20 000-dalton light chain and analyzed the data by Perrin–Weber treatment to get an estimate of the rotational correlation time of the molecule. The rotational correlation time estimated for the AEDANS-labeled light chain bound to the S-1 heavy chain indicates a considerably faster rate of molecular rotation than would be expected for a molecule of the size and shape of smooth muscle myosin S-1. An interesting finding is that the binding of the labeled light chain to 6S myosin resulted in only a slight increase in the rotational correlation time from 16.8 to 21.2 ns while the rotational correlation time of the AEDANS conjugate of the light chain associated with 10S myosin was significantly larger than those obtained with S-1 and 6S myosin (Table II). It therefore seems likely that the structural transition of smooth muscle myosin from 6 S to 10 S brings about a conformational change in the region of the light-chain binding site(s) of myosin

which results in a less degree of local rotational mobility of the 20 000-dalton light-chain segment to which the probe is bound. It has been proposed that the 10S–6S transition of smooth muscle myosin or some component associated with this transition may be important for the regulation of smooth muscle actomyosin function. An attempt has been made to identify such a component, and it has been suggested that the change in the conformation at the S-1/S-2 junction is the determinant for regulation of smooth muscle actomyosin ATPase activity (Ikebe & Hartshorne, 1984, 1985a; Ito et al., 1989; Higashihara et al., 1989; Higashihara & Ikebe, 1990; Hartshorne et al., 1988). Location of the regulatory light chains of myosin is not yet known in detail, but consideration of the available structural data does provide a basis for speculation. It has been suggested by electron microscopy and cross-linking studies that regulatory light chains of various types of myosins are located at or near the head–rod junction, whose region is known to have a thin and flexible structure (Craig et al., 1980; Katayama & Wakabayashi, 1982; Wallimann et al., 1982; Flicker et al., 1983; Winkelman et al., 1983). Consistent with the foregoing notion is a recent observation using a bifunctional photoprobe that Cys-108 of the 20 000-dalton light chain can be cross-linked to sites in the skeletal myosin heavy chain located over 95 000 daltons away from the N-terminus in the primary structure (Morita et al., 1989). This is likely to be a region near the S-1/S-2 junction. Thus, together with the aforementioned reports, the present work suggests that the 10S–6S transition is at least in part correlated with changes in the local rotational motion of the light chain segment which is associated with the heavy chain at or near the head–rod junction. Previously, it was pointed out that the HMM heads assumed a different orientation in a phosphorylation-dependent manner that led to a change in the ATPase activity of HMM; i.e., the heads of dephosphorylated HMM were bent toward the tail axis while those of phosphorylated HMM were more extended (Suzuki et al., 1985; Hartshorne & Ikebe, 1987). However, the change in the head orientation of HMM by phosphorylation is not extreme, and a significant portion of dephosphorylated HMM still shows an extended form. Also present are a significant number of phosphorylated HMM heads bent toward the tail axis. Therefore, it is unlikely that the ATPase activity is determined by the head orientation. The present work showed that the segmental flexibility at the head–rod junction changes by light-chain phosphorylation and therefore it appears likely that an apparent change in the orientation of the myosin heads is accompanied by the change in the segmental flexibility. Although further investigation is required, the changes in conformation at the head–rod junction can, in part, account for regulation of the smooth muscle actomyosin ATPase activity.

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