Novel Sensors on the Regulatory Switch on the Regulatory Light Chain of Smooth Muscle Myosin*

Sam M. Mazhari, Curtis T. Selser, and Christine R. Cremo‡

From the Department of Biochemistry, University of Nevada, Reno, Nevada 89557

Smooth muscle myosin can be switched on by phosphorylation of Ser-19 of the regulatory light chain. Our previous photocross-linking results suggested that an element of the structural mechanism for the regulatory switch was a phosphorylation-induced motion of the regulatory light chain N terminus (Wahlstrom, J. L., Randall, M. A., Jr., Lawson, J. D., Lyons, D. E., Siems, W. F., Crouch, G. J., Barr, R., Facemyer, K. C., and Cremo, C. R. (2003) J. Biol. Chem. 278, 5123–5131). Here we used three different approaches to test this notion, which are reactivity of cysteine thiols, pyrene and acrylodan spectral analysis, and pyrene fluorescence quenching. All methods detected significant differences between the unphosphorylated and phosphorylated regulatory light chain N termini in heavy meromyosin, a double-headed subfragment with an intact regulatory switch. These differences were not observed for subfragment-1, a single-headed, unregulated subfragment. In the presence of either ATP or ADP, phosphorylation increased the solvent exposure and decreased the polarity of the environment about position 23 of the regulatory light chain of heavy meromyosin. These phosphorylation-induced structural changes were not as evident in the absence of nucleotides. Nucleotide binding to unphosphorylated heavy meromyosin caused a decrease in exposure and an increase in polarity of the N terminus, whereas the effects of nucleotide on phosphorylated heavy meromyosin were the opposite. We showed a direct correlation between the kinetics of nucleotide binding/turnover and the conformational change reported by acrylodan at position 23 of the regulatory light chain. Acrylodan-A23C also reports the heads up (extended) to flexed (folded) transition in unphosphorylated heavy meromyosin. This is the first demonstration of direct coupling of nucleotide binding to conformational changes in the N terminus of the regulatory light chain.

Smooth muscle myosin (SMM) and nonmuscle myosin are hexameric motor ATPases of ~500 kDa mass composed of pairs of HC, ELC, and RLC. A globular motor domain (N-terminal HC) and a light chain binding domain (HC + ELC + RLC) form a head (S1), and the two heads are dimerized by the HC C-terminal halves, which form a coiled-coil. The motor domain contains the catalytic site and the actin binding site (1). Only the head domain and its subfragments have been crystallized to date so there are no atomic resolution structures of a double-headed construct.

The act-activated ATPase activity and motor properties of SMM and nonmuscle myosin are regulated by phosphorylation of Ser-19 of the RLC, which is greater than 10 nm from the catalytic site (1–4). Phosphorylation enhances the ATPase activity by more than 1000-fold at saturating actin concentrations (5, 6). Domain requirements for regulation have been elucidated through studies of various proteolytic and expressed subfragments of SMM and nonmuscle myosin. HMM, which lacks the C-terminal two-thirds of the tail, is double-headed and regulated (5–7), but expressed HMM constructs with truncated tails too short to form stable double-headed structures are unregulated (8–10) as is S1 (7, 11, 12) and single-headed myosin (13, 14). Furthermore, a nonmuscle HMM construct without one of the motor domains but with both regulatory domains is not regulated (15). Therefore, two full heads connected together with enough coiled-coil to allow for dimerization are required for regulation.

Removal of the RLC abolishes regulation (16), but the ELC can be removed with retention of partial regulation (10, 17). Site-specific, chimeric, and deletion mutations of the RLC have shown that the H-helix (18) in the C-terminal lobe (19) and the N terminus (20, 21) are critical for regulation. Unfortunately, none of the crystal structures contain coordinates for the RLC N terminus (residues 1–24), presumably due to its lack of ordered secondary structure in single-head constructs. Our photocross-linking studies (22, 23) provide structural information about the RLC N terminus in UP-HMM. Our data (23) suggest that phosphorylation of Ser-19 causes a large movement of the N terminus from an extended to a folded conformation. The UP N terminus may stabilize the RLC-RLC or RLC-S2 interaction that allosterically down-regulates the ATPase activity. This model is consistent with a progressive increase in ATPase for UP-SMM as residues are sequentially deleted from the N terminus (21).

Because our previous studies did not provide specific information about the phosphorylated state of HMM, we have turned to alternative approaches to further understand the role of the N terminus in the structural mechanism of the regulatory switch. In this study we used reactivity of cysteines, pyrene acrylamide quenching, and fluorescence spectral analysis

© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.

This paper is available online at http://www.jbc.org

Received for publication, June 24, 2004
Published, JBC Papers in Press, July 15, 2004, DOI 10.1074/jbc.M407062200

The abbreviations used are: SMM, smooth muscle myosin; HMM, heavy meromyosin; RLC, regulatory light chain; ELC, essential light chain; Pi, inorganic phosphate; UP-NON, UP-ATP, or UP-ADP-HMM, unphosphorylated heavy meromyosin; UP-NON, UP-ATP, or UP-ADP-HMM, unphosphorylated heavy meromyosin with no nucleotides, ATP, or ADP, respectively; HC, heavy chain; RLC, regulatory light chain; ELC, essential light chain; S1, subfragment-1; S2, N-terminal portion of the rod; FTP, formycin triphosphate; FDP, formycin diphosphate; ATPγS, adenosine-5'-O-(thiophosphate); ACR, acrylodan; IAF, 5-iodoacetamidofluorescein; TMR, tetramethylrhodamine-5-iodoacetamide dihydroiodide; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol.
of both pyrene and acrylodan to study conformational changes about the RLC affected by RLC phosphorylation and nucleotide binding. We focused on the interactions near position 23, where significant conformational differences between the UP-HMM and P-HMM states were found. These differences were most apparent when ADP or ATP was bound to the active site. Such differences were not found in the unregulated S1, thus directly implicating them in the regulatory structural mechanism. Using transient methods we showed that the RLC N-terminal conformational changes occurred at rates paralleling the nucleotide binding and P release. This is the first instance of a probe in the regulatory domain reporting nucleotide binding in the motor domain. The possible role of the observed conformational changes to the regulation mechanism of SMM is discussed.

MATERIALS AND METHODS

Reagents—Common reagents were purchased from Sigma or Fisher. ATP, ADP, Staphylococcus aureus protease (V8), bovine brain catalase, bovine plasma thrombin, Ellman’s reagent, lactate dehydrogenase, pyruvate kinase, 2.5 mM phospho(enol)pyruvate, 25 μM actin, and 0.6 or 0.1 mg/ml of UP- or P-HMM, respectively. The reaction (25 °C) was started by adding MgATP. Reactions lacking HMM were used for background subtraction.

Reactivity of RLC Thiols—The conditions of the thiol reactivity experiments were similar to those used previously (33–35). UP- or P-HMM/SmS (0.75–2.5 mg/ml; 100–200 μM in reaction buffer (50 mM MOPS (pH 7.0), 50 mM KCl, 1 mM EGTA, 2 mM MgCl₂, and 50 μM DTT with or without ATP or ADP) was incubated at 25 °C with 500 μM IAF. Aliquots (13 μg) were quenched at 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5 min by adding 10 μl of reducing gel sample buffer (2×, 0.5 M DTT). To measure reaction stoichiometry, IAF was raised to 600 μM at 3 min, and an aliquot was collected at 4 min. Samples were analyzed on Bis-Tris PAGE gels (Invitrogen). Excess IAF was removed, and proteins were separated from unlabeled gels overnight in 300 ml of 50% methanol, 10% glacial acetic acid. Gels were washed twice in 50 mM MOPS (pH 7.0) for 30 min before fluorescence imaging with a Vis-Blue plate (~480 nm), 500–630 nm band-pass filter, and a transilluminator (UVP gel-documenting system, Upland, CA) (Fig. 2A). Imaged gels were stained with Coomassie Blue (36), destained, and further imaged with white light and no filters (Fig. 2B). The normalized fluorescence of the RLC bands was calculated by dividing the fluorescence by the Coomassie densities (Labworks software, UVP) to correct for variable protein loading. Because [IAF] >> [accessible protein-SH], the rate constants for the reaction (k, min⁻¹) were calculated by fitting Equation 1 for a first order reaction (37).

\[ [F]_t = [F]_0 + [A], [1 - exp(-kt)] \]  

where \( [F]_t \) was labeled RLC at time \( t \), \( [F]_0 \) = the y intercept, and \([A]\_0\) = total available RLC-Cys (amplitude).

Actin-activated ATPase by Single Turnover of FTP—All single turnovers (6) were performed in 10 mM MOPS (pH 7.0), 0.2 mM EGTA, 1 mM DTT, 2 mM MgCl₂, 50 mM NaCl, 200 μM ATP, and 5 μM actin at 25 °C using a Hi-Tech SF-61 DX2 stopped-flow spectrophotometer equipped with a 75-watt mercury-xenon lamp with an excitation wavelength of 313 nm (bandwidth = 4 nm) and a 365–453 nm emission band-pass filter (P70–400, Thermocor). Regulated preparations showed a monophasic turnover rate of 0.0008–0.0002 s⁻¹ at 5 μM actin. Unregulated preparations (apparently due to exposure to an unknown component in some lots of diisopropyl fluorophosphate) showed a fast phase of variable rate and amplitude in addition to the regulated rate and were not used. The rate constants (k) and corresponding amplitudes (ΔF) were calculated as described (38).  

Nucleotide Binding Kinetics—Experiments were performed with the same instrument and buffer as the single turnovers except actin was not present, and ATP or ADP was added as indicated. To measure FTP binding kinetics, 2 μM FTP was mixed with 200 μM chicken gizzard (MLRN_HUMAN, accession P24844) heavy chains, and formycin fluorescence was monitored. For ACR fluorescence, 2 μM A23C/RLC HMM heads was mixed with either FTP, ATP, or ADP with excitation at 404 nm (bandwidth 4 nm) and an emission long-pass filter of >455 nm (GG455, Schott Glass). Data analysis was performed using GraphPad Prism (San Diego, CA).

RESULTS

We were interested in detecting structural differences that correlate to differences in regulatory properties of UP- and P-HMM. Our approach was to prepare a series of HMM-bound or S1-bound single-Cys RLC mutants and use these in a variety of chemical and spectral experiments designed to detect conformational changes. S1 is not regulated by phosphorylation and, therefore, serves as a negative control for structural changes not related directly to regulation. Large conformational changes occur in myosin during the nucleotide hydrolysis cycle, so all measurements were both in the absence (NON) and presence of the nucleotides MgATP or MgdAP. Thus, measurements were made under six different states, three UP states, UP-NON, UP-ATP, UP-ADP, and three phosphorylated states, P-NON, P-ATP, P-ADP for HMM and S1. Fig. 1A shows our homology model of the regulatory domain (23), and Fig. 1B shows the RLC sequence with substituted cysteines. Surface polar uncharged positions were chosen based on our previous work (22), which implicated them in RLC-RLC interactions; Q15C, A23C, and the native Cys-108, when labeled with a photocross-linker and irradiated, formed RLC-RLC cross-links in UP-HMM but not in P-HMM. S9C was unable to form...
unregulated protein (6). The turnover rate for freshly prepared
unlike the steady-state method, can detect small amounts of
ATPase activities by single-turnover of FTP (Table I), which
shown). To quantify regulation, we measured actin-activated
ELC, and RLC as measured by gel densitometry (data not
All HMM preparations contained equimolar amounts of HC,
change procedure shown to yield fully regulated HMM (6, 22).
RLC in HMM and S1. We used a thoroughly tested RLC ex-
required exchange of mutant RLC, one at a time, for native
phosphorylation site for activation of SMM (1, 2).
T9C, Q15C, and A23C, flank Ser-19, which is the phos-
are involved in RLC-
cross-links (22, 23). T9C and T83C may be involved in RLC-
Actin-activated ATPase Measurements—Our methodology
required exchange of mutant RLC, one at a time, for native
RLC in HMM and S1. We used a thoroughly tested RLC ex-
change procedure shown to yield fully regulated HMM (6, 22).
All HMM preparations contained equimolar amounts of HC,
ELC, and RLC as measured by gel densitometry (data not
shown). To quantify regulation, we measured actin-activated
ATPase activities by single-turnover of FTP (Table I), which
unlike the steady-state method, can detect small amounts of
unregulated protein (6). The turnover rate for freshly prepared
UP-HMM was monophasic (0.0014 s−1; Table I) and showed an
increase in activity upon phosphorylation (~175×, at 5 μM
actin), consistent with our previous work (6, 38). The turnover
for aged UP-HMM was biphasic, with a first phase ~10× faster
than a second phase of normal rate. The weighted average rate
was ~7× that of the control. The turnover rates of the mutants
(similar in age to the aged unmodified) ranged between the
aged and freshly prepared unmodified HMM. Therefore, we
concluded that the slightly altered rates were due to the age of
the protein, not to the mutation/thiol modification.
Turnovers for pyrene-labeled HMM could not be measured
because pyrene interfered with the FTP signal. To approximate
the turnover kinetics of HMM that was fully labeled with a
bulky group similar to pyrene, we analyzed TMR-A23C HMM
from a large probe. However, exchange efficiencies for all py-
ene- or ACR-labeled preparations were ~50%, so less than
this maximal effect can be predicted for pyrene-labeled HMM.
Under these conditions the ACR-A23C data were similar to the
ACR-A23C-ACR = 40–60% exchanged.

|                  | Unphosphorylated | Phosphorylated |
|------------------|------------------|----------------|
|                  | k         | ΔF          | k         | ΔF          |
| Native (fresh)a  | 0.0014   | 100         | 0.31     | 78          |
| Phase1           | 0.0014   | 100         |          |             |
| Phase2           | 0.0018   | 61          | 0.021    | 12          |
| Phase3           | 0.0100   | 100         | 0.28     | 100         |
| WT Ave.          | 0.0024   | 100         |          |             |
| Native (aged)b   | 0.0240   | 34          | 0.39     | 83          |
| Phase1           | 0.0212   | 66          | 0.027    | 17          |
| Phase2           | 0.0087   | 100         | 0.33     | 100         |
| Phase3           | 0.0097   | 15          | 0.30     | 61          |
| Phase4           | 0.0012   | 85          | 0.04     | 39          |
| Phase5           | 0.0025   | 100         | 0.20     | 100         |
| Phase6           | 0.045    | 67          | 0.47     | 84          |
| Phase7           | 0.0041   | 33          | 0.027    | 16          |
| Phase8           | 0.032    | 100         | 0.39     | 100         |
| HMM              | 0.023    | 22          | 0.035    | 22          |

a Unmodified native HMM analyzed immediately after preparation or stored 1–3 days at 0 °C.
b Weighted average (WT Ave.) for a biphasic fit was (k1ΔF1 + k2ΔF2) where k1 and k2 = rate constants, and ΔF1 and ΔF2 = % of total
amplitude corresponding to each k. Total amplitude ~ ΔF1 + ΔF2 = 100%.
c Unmodified native HMM stored 13–16 days at 0 °C. This is a reference for the modified samples that were aged a similar length of time.
d T9C, A23C, and A23C-TMR ~ 90–100% exchanged; A23C-ACR = 40–60% exchanged.

Fig. 1. Homology model of the regulatory domain of SMM. A, homology model of the regulatory domain of SMM (23). Green, ELC;
gray, HC (residues Arg-787–Leu-850); blue, RLC (residues Phe-25–
Gly-165). Ser-59, Thr-83, and Cys-108 along with Phe-25, the first
represented N-terminal residue of the RLC, are shown as red sticks. B, native amino acid sequence of RLC. Mutated
residues are shown in bold and marked with open stars. Cys-108 (solid
star) was mutated into Ala. The italicized amino acids are not in the
homology model (high mobility regions in the crystal structures (3)).
The 8 α-helices, A–H, are marked with lines. Lines above are predicted
by our homology model (A), and lines below correspond to the ones
predicted by GOR IV program (64).  
IAF, and the rate and extent of the reaction was quantitated (Fig. 2). Fig. 2A shows the fluorescence image from an experiment with A23C/RLC UP-HMM revealing that both the mutant RLC and the native ELC incorporate significant IAF, but the HC does not. The Coomassie Blue-stained image (Fig. 2B) shows that IAF-RLC emerges as a band immediately above the RLC. Fig. 2C shows the normalized fluorescence versus time for the RLC with a fit to a single-exponential function (line). A 100%-labeled RLC mutant (not HMM-bound; F.R.) was used to calibrate the stoichiometry. At 4 min and 0.6 mM IAF either <3% (Cys-108 and S59C) or >80% (T9C, Q15C, A23C, T83C, T383C) of the RLC was modified with IAF for all HMM nucleotide and phosphorylation states (data not shown). Therefore, structural changes induced by nucleotide or phosphorylation to strongly shield an exposed residue or to expose a strongly shielded one were not detected with this method.

Table II summarizes all the thiol labeling rate constants \( k \). Mutants fell into four rate classes; Class 1 was unreactive, Class 2 showed no significant effect of nucleotide in either the UP or P states, Class 3 showed rates too fast to measure, and finally, Class 4 showed a significant effect of nucleotides only in UP-HMM. The measurable rates for the P states were slower than the UP states of both HMM and S1 for Class 2, 3, and 4, and the effect was more pronounced for the three N-terminal residues. Because this effect was also true for S1, we do not consider this relevant to regulation. The P group may suppress thiol ionization or electrostatically repulse the negatively charged IAF.

The RLC of native HMM or S1 (Cys-108) fell into the unreactive class. Fig. 1 suggests that the unreactivity is due to an interaction of the side chain with the S1 HC. It is unlikely that this residue is shielded by RLC-RLC or RLC-S2 interaction as it is also unreactive in S1. The lack of reactivity of Cys-108 in native HMM and S1 allowed study of mutant HMMs with less than 100% RLC exchange. UP/P RLC/S59C HMM was also unreactive. Fig. 1A indicates that the side chain of Ser-59 is solvent-accessible, but the proximal Asp-55 (within 0.6 nm) likely suppresses ionization to the thiolate anion (39, T83C and T9C fell into Class 2 as both showed measurable thiolabeling rates that were not significantly sensitive to the presence of nucleotides. T83C is near the amino end of helix D with its side chain solvent exposed.

Q15C showed the fastest reaction rates, which may suggest higher flexibility for this region. This is supported by secondary structure prediction (Fig. 1B) and our modeling work (23). Position 15 is in the region known to be critical to myosin light chain kinase function (40, 41). Lack of intrinsic structure in proteins is often associated with regions involved in intrmolecular interactions (42, 43); therefore, it may be thermodynamically necessary for kinase binding. The large difference in the reactivity of T9C and A23C in comparison to Q15C also indicated that the former two positions may be located within ordered regions, which is in agreement with our modeling work (23).

A23C fell into Class 3, as reaction rates were significantly sensitive to the presence of nucleotides but only in UP-HMM. Neither P-HMM nor UP- or P-S1 rates was significantly sensitive to nucleotides, although the trend was downward. This unique pattern suggested that A23C reactivity is sensitive to a regulation-related parameter. Therefore, this mutant was further investigated using additional approaches designed to monitor conformational changes.

Effect of IAF-RLC Conjugation upon HMM Regulation—It is known that modification of the HC at the relatively reactive Cys-717 (SH1) can alter smooth HMM regulation (44). Even though we observed a low level of HC modification (Fig. 2; <6% of fully reacted RLC at 2.5 min; data not shown), it was important to ensure that HMM remained regulated during the IAF reaction. Fig. 3 shows that the steady-state actin-activated MgATPase of native HMM was not significantly altered after reaction with IAF. The single-turnover method could not be used because IAF masks FTP fluorescence.

Thiol Reactivity of the ELC—The ELC incorporated IAF to as much as 65% of the fully labeled RLC after 4 min at 0.6 mM IAF. This high reactivity is expected from the homology model (Fig. 1A), which indicates that 3 of the 4 Cys residues are surface-exposed with potentially flexible secondary structure (residues 1, 84, and 137 (45)). This relatively high level of IAF incorporation into the ELC did not disrupt HMM regulation (Fig. 3), consistent with other studies (10, 46). The rates of ELC incorporation did not appear to be sensitive to nucleotide binding and phosphorylation (data not shown).

Fluorescence Quenching of Pyrene-labeled A23C/RLC HMM and S1—Fluorescence acrylamide quenching of pyrene, a neutral environment-sensitive fluorescent probe, was used to evaluate solvent accessibility about A23C/RLC. Fig. 4 shows the Stern-Volmer plots of pyrene-A23C UP-HMM with/without ATP as examples of typical quenching data. Plots for all states of HMM or S1 were linear \( R^2 > 0.97 \), indicating a single population of the probe (data not shown). Table III summarizes the Stern-Volmer quenching constants \( K_{SV} \) of the pyrene-A23C/RLC on both HMM and S1. The \( K_{SV} \) values for HMM or S1 were 3–6-fold lower (less solvent accessibility) than those for pyrene-mercaptoprotochlorophyll \( K_{SV} = 2.5 \, \text{m}^{-1} \); data not shown. UP- and P-HMM had the same \( K_{SV} \) in the absence of nucleotide \((-0.59 \, \text{m}^{-1})\), but in the presence of either ATP or ADP, the \( K_{SV} \) values were different by ∼100%, with the P-HMM being more exposed. UP-HMM with nucleotide had a 27% lower \( K_{SV} \) value than UP-HMM without nucleotide, and P-HMM with nucleotide had a 34% higher \( K_{SV} \) value than P-HMM without nucleo-
otide. Therefore, nucleotide caused a decrease in solvent exposure for UP-HMM but an increase in solvent exposure for P-HMM. In contrast to HMM, the solvent exposure in S1 was essentially the same in the six different states. The average $K_{SV}$ value of 0.86 m$^{-1}$ was higher than any of the HMM states, ~7% higher than the nucleotide-bound P-HMM. Therefore, the pyrene on S1 is slightly more exposed than P-HMM but cannot sense either nucleotide binding or phosphorylation. The differences between S1 and HMM suggest that the observed nucleotide- and phosphorylation-dependent differences in solvent exposure for the regulated HMM are correlated to structural changes brought about by either S1-S1 or S1-S2 interactions. These interactions are important in maintaining the "off" state of the HMM and are minimized in nucleotide-bound P-HMM.

**Fluorescence Spectroscopy of Pyrene-labeled A23C/RLC HMM and S1**—The emission intensity of pyrene is increased in a less polar and decreased in a more polar environment. Fig. 5 shows fluorescence emission spectra for pyrene-A23C/RLC HMM. The $\lambda_{max}$ and intensity data for these spectra (Fig. 5, A and B) or for S1 spectra (not shown) are summarized in Table III. The excitation and emission $\lambda_{max}$ were 344 ± 1 (not shown) and 383 ± 1 nm (Table III), respectively, and the spectral shapes were the same for all measurements (see Fig. 5). In the absence of nucleotide, the P-HMM (Fig. 5B) had a lower intensity than the UP-HMM (Fig. 5A). This could be brought about by either enhanced solvent accessibility or as a consequence of increase in the polarity of the environment brought about by either charged groups or fewer nonpolar groups. The latter could be the consequence of the close proximity of the phosphate to the pyrene group. ATP and ADP had the opposite effects on the fluorescence intensities of UP-NON versus P-NON-HMM. ATP and ADP reduced the intensity of the UP-NON state by 10 and 15%, respectively, indicating enhanced
polarity of the environment. In contrast, the fluorescence intensity of P-HMM was increased by ATP and ADP by 27 and 18%, respectively, indicative of decreased polarity upon nucleotide binding. In contrast, P-HMM remains in a more nonpolar environment (elevated in intensity upon nucleotide binding for UP-HMM. In contrast, P-HMM was sensitive to nucleotide binding in the above equilibrium measurements. To confirm this finding and to measure the ACR-A23C/RLC fluorescence intensity in UP- or P-S1; however, phosphorylation caused an ~8% increase in the fluorescence of all three nucleotide states, indicative of reduced polarity. The overall nucleotide- and phosphorylation-sensitive changes in intensities of the pyrene-A23C in HMM and the relative insensitivity in S1 mirror the pattern of the acrylamide quenching data (Table III).

Fluorescence Spectroscopy of ACR-labeled A23C Mutant—ACR, like pyrene, is an environment-sensitive probe (29) that commonly exhibits blue-shifted, increased fluorescence in a more nonpolar environment and red-shifted, decreased fluorescence in a more polar environment (47–49). The fluorescence emission spectra for ACR-A23C/RLC HMM (Fig. 6, A and B) or S1 (not shown) were measured, and the $\lambda_{max}$ and intensities are reported in Table III. The excitation $\lambda_{max}$ for all samples was ~388 ± 2 nm, which was considerably red-shifted from ~360 ± 2 nm for ACR-conjugated mercaptoethanol (data not shown), indicating a less polar environment for the conjugated ACR, as expected. Similarly, the emission $\lambda_{max}$ for HMM (Fig. 6, A and B, and Table III) and S1 (Table III) in all 6 states (491–502 nm) were considerably blue-shifted from their corresponding value of ~530 nm for ACR-mercaptoethanol (data not shown). For HMM without nucleotides, the emission intensity and the $\lambda_{max}$ were unaffected by phosphorylation. The addition of nucleotides to UP-HMM caused an ~8-nm red shift and 18% reduction in intensity. This is in agreement with the pyrene spectral data suggesting a transition to a more polar environment upon nucleotide binding for UP-HMM. In contrast, P-HMM remains in a more nonpolar environment (elevated intensity and blue-shifted $\lambda_{max}$) similar to NON-UP-HMM regardless of the nucleotide state. These data show that nucleotide must be present to observe a difference between UP- and P-HMM, as was found for pyrene acrylamide quenching. In contrast, S1 showed no significant differences in $\lambda_{max}$ or intensities (<3%) for the 6 states. Note that intensities between HMM and S1 cannot be compared to one another. Readings shown are from one preparation. Errors on these measurements are approximately 5%.

Values are ± approximately 2 nm due to the broad spectra.

Fig. 5. Steady-state emission spectra of pyrene-labeled A23C/RLC UP and P-HMM. Uncorrected spectra were collected at 25 °C with a Spex Fluorolog spectrofluorometer equipped with a 150-watt xenon lamp in the absence (NON) and presence of ATP (0.5 mM) or ADP (1 mM). Excitation was at 342 nm with a 1-nm bandwidth. Emission bandwidth was 4.5 nm. Each sample contained 0.5 μM HMM in fluorescence buffer (see Fig. 4). A, unphosphorylated, and B, phosphorylated.

## Table III

| Nucleotide state | HMM | S1 |
|-----------------|-----|----|
|                 | Unphosphorylated | Phosphorylated* | Unphosphorylated | Phosphorylated* |
| $k$ Values for thiol reactivity (min$^{-1}$) | | | | |
| NON* | 2.5 ± 0.3 | 0.8 ± 0.1 | 2.7 ± 0.7 | 1.6 ± 0.2 |
| ATP | 1.2 ± 0.3 | 0.4 ± 0.2 | 2.9 ± 0.5 | 1.2 ± 0.5 |
| ADP | 1.3 ± 0.3 | 0.5 ± 0.2 | 2.3 ± 0.2 | 1.1 ± 0.4 |

K$_q$ for acrylamide quenching of pyrene (m$^{-1}$)

| State | NON | ATP | ADP |
|-------|-----|-----|-----|
| NON   | 0.59 ± 0.03 | 0.58 ± 0.02 | 0.85 ± 0.01 |
| ATP   | 0.43 ± 0.05 | 0.78 ± 0.04 | 0.83 ± 0.01 |
| ADP   | 0.42 ± 0.03 | 0.82 ± 0.07 | 0.83 ± 0.01 |

Values are from Table II.

* NON, no nucleotide added.

Stern-Volmer quenching constants (K$_q$) of HMM and their S.E. reflect three separate experiments using two independent protein preparations.

See Fig. 6 and 7 for details regarding the collection of spectra. Readings shown are from one preparation, but replicate measurements on one other preparation were essentially the same (range ± 5%).

All HMM samples were the same fluorophore concentration, and therefore, the emission intensities can be directly compared. This is also true for S1. However, the HMM and S1 cannot be compared to one another. Readings shown are from one preparation. Errors on these measurements are approximately 5%.

Values are ± approximately 2 nm due to the broad spectra.

### Summary of data for A23C/RLC HMM and S1

| Emission spectra of pyrene* |
|----------------------------|
| Emission intensity | $\lambda_{max}$ |
|-----------------|-----------|
| NON | 383 | 71.8 | 383 |
| ATP | 383 | 91.8 | 383 |
| ADP | 383 | 80.4 | 383 |

| Emission spectra of ACR* |
|-------------------------|
| Emission intensity | $\lambda_{max}$ |
|-----------------|-----------|
| NON | 492 | 75.8 | 492 |
| ATP | 492 | 74.4 | 492 |
| ADP | 492 | 70.8 | 492 |

### Table III

| Nucleotide state | HMM | S1 |
|-----------------|-----|----|
|                 | Unphosphorylated | Phosphorylated* | Unphosphorylated | Phosphorylated* |
| $k$ Values for thiol reactivity (min$^{-1}$) | | | | |
| NON* | 2.5 ± 0.3 | 0.8 ± 0.1 | 2.7 ± 0.7 | 1.6 ± 0.2 |
| ATP | 1.2 ± 0.3 | 0.4 ± 0.2 | 2.9 ± 0.5 | 1.2 ± 0.5 |
| ADP | 1.3 ± 0.3 | 0.5 ± 0.2 | 2.3 ± 0.2 | 1.1 ± 0.4 |

K$_q$ for acrylamide quenching of pyrene (m$^{-1}$)

| State | NON | ATP | ADP |
|-------|-----|-----|-----|
| NON   | 0.59 ± 0.03 | 0.58 ± 0.02 | 0.85 ± 0.01 |
| ATP   | 0.43 ± 0.05 | 0.78 ± 0.04 | 0.83 ± 0.01 |
| ADP   | 0.42 ± 0.03 | 0.82 ± 0.07 | 0.83 ± 0.01 |

Values are from Table II.

* NON, no nucleotide added.

Stern-Volmer quenching constants (K$_q$) of HMM and their S.E. reflect three separate experiments using two independent protein preparations.

See Fig. 6 and 7 for details regarding the collection of spectra. Readings shown are from one preparation, but replicate measurements on one other preparation were essentially the same (range ± 5%).

All HMM samples were the same fluorophore concentration, and therefore, the emission intensities can be directly compared. This is also true for S1. However, the HMM and S1 cannot be compared to one another. Readings shown are from one preparation. Errors on these measurements are approximately 5%.
the rate of the fluorescence change relative to the rate of nucleotide binding, ACR-A23C/RLC UP-HMM and either ATP or ADP were rapidly mixed in a stopped-flow fluorometer, and the resulting ACR fluorescence changes were recorded over time (Fig. 7). As expected from the data in Table III, the intensity of ACR fluorescence drops as nucleotide binds. The amplitude of the drop depended upon the amount of nucleotide added and is greater for ATP than ADP due to the lower $K_d$ of ATP (50). Under conditions where all the catalytic sites were occupied (2.5 $\mu$M ATP), the maximal amplitude was 8–9%. The amplitudes of 22% (0.5 $\mu$M ADP) and 45% (2.5 $\mu$M ADP) of maximal are in reasonable agreement with the expected values calculated from the $K_d$ for ADP (1.2 $\mu$M) (50). These data demonstrated the expected concentration- and affinity-dependent decrease in the ACR fluorescence due to ATP and ADP. A complete analysis of the ACR kinetics is in progress.

The kinetics of ACR fluorescence and nucleotide turnover in HMM were compared and show that the two events are correlated in time. Nucleotide binding was monitored using the fluorescent analogue FTP which increases in fluorescence by $\sim 250\%$ upon binding to the active site (6, 38). Fig. 7A shows the transient for FTP binding to ACR-A23C/RLC HMM (FTP stoichiometric to sites) along with the ACR fluorescence transient. As the FTP binds and its fluorescence increases ($\sim 270\%$), the ACR fluorescence decreases ($\sim 7\%$). Rates for the first 15 s, where FTP binding rate $\gg$ FDP $+$ P$_i$, release rate were found to be within a factor of 2, suggesting that ACR at RLC/A23C reports nucleotide binding. As expected from the spectral studies, P-HMM (Fig. 7B) showed no significant changes in ACR fluorescence upon FTP binding (less than 1.5%). Fig. 8C is similar to Fig. 7A except that FTP and ACR fluorescence are monitored for a complete turnover. The FTP signal mirrors the ACR signal throughout the FTP binding and subsequent turnover and release of FDP + P$_i$. A fit to the region where FDP + P$_i$ release $\gg$ FTP binding (dotted lines) shows that the FDP release rate (limited by P$_i$ release) and the ACR signal have essentially the same rate constant. These combined data (Table III, Figs. 7 and 8) show that the ACR signal observed for UP-HMM reports nucleotide binding and release. This change in signal was not observed in P-HMM.

**DISCUSSION**

Our previous results suggested that an element of the structural mechanism for the regulatory switch was a phosphorylation-induced motion of the RLC N terminus. In this study using three different techniques, reactivity of thiolos to IAF, pyrene and ACR spectral analysis, and pyrene fluorescence quenching we have provided further evidence consistent with this notion. We have detected significant differences in the structure of the RLC N-terminal domain in the ATP- and ADP-bound states of UP and P-HMM that were largely absent in nucleotide-free HMM and S1. These studies show for the first time that nucleotide binding at the motor domain can be sensed at the RLC N terminus and that the sensing mechanism requires a double-headed fragment.

**Phosphorylation Increased the Exposure of the RLC N Terminus in Nucleotide-bound HMM—Acrylamide quenching data for the A23C/RLC showed that HMM but not S1 was sensitive to phosphorylation, and this sensitivity required the presence of ATP or ADP. For HMM without nucleotides, the solvent exposure of this site is an intermediate value and is the same for UP and P states. However, with nucleotides, the accessibility to the solvent for UP-HMM is $2 \times$ lower than that for P-HMM. This suggests that, with nucleotides, the A23C position changes conformation from a less solvent-exposed position upon phosphorylation. Two heads are required for this conformational change to occur, because all states of S1 were similar and resembled P-HMM. The lower exposure in UP-HMM may, therefore, be due to RLC-RLC interactions, which would be consistent with our previous photocross-linking studies showing that benzophenone-labeled A23C cross-links between the two RLCs only in UP-HMM, not in P-HMM (25).

These observations suggest a model in which there are two conformations of the RLC N terminus of HMM in rapid equilibrium; the “on” conformation, with a more solvent accessible N terminus ($K_{SV} \sim 0.8 \text{ M}^{-1}$; phosphorylated with nucleotide), and the off conformation, with a more buried RLC N terminus ($K_{SV} \sim 0.4 \text{ M}^{-1}$; UP with nucleotide). A slow interconversion between these two conformations would have produced nonlinear Stern-Volmer plots, which were not observed. Assuming that these two nucleotide-bound states reflect 100% on or off, the intermediate $K_{SV}$ value of $\sim 0.59 \text{ M}^{-1}$ for the nucleotide-free states suggests that the two conformations are present in approximately equivalent amounts, and phosphorylation has no effect upon their relative stability. Nucleotide binding drives the equilibrium to the off conformation in UP-HMM and the on conformation in P-HMM. S1 (in all states) behaves similarly to P-HMM, suggesting that the heads act independently in P-

![Fig. 6. Steady-state emission spectra of ACR-labeled A23C/RLC HMM in UP and P states.](Image)

![Fig. 7. Transients of nucleotide binding and ACR fluorescence for ACR-labeled A23C/RLC UP-HMM.](Image)
HMM. This model is very similar to a recent model proposed for scallop myosin (51), which is regulated by calcium binding to the regulatory domain; two conformations are predicted in which nucleotide and calcium are allosteric effectors with calcium favoring the on conformation and ADP favoring the off conformation. Co-operative switching between off and on states occurs only for calcium binding in the presence of ADP (51–53).

Whether the smooth HMM case involves cooperative switching will require quenching measurements of HMM with only one phosphorylated head. This molecule shows a largely down-regulated behavior in the presence of nucleotides, suggesting that there is cooperativity between the heads (6). The ACR spectral data and to some extent the pyrene spectral data as discussed below support the model proposed here.

Phosphorylation Decreases the Polarity of the Environment of the RLC N Terminus in Nucleotide-bound HMM—The emission spectra of pyrene-labeled and ACR-labeled A23C/RLC showed the same pattern found for the thiol labeling and the acrylamide quenching data, which leads us to believe that the spectral changes are reporting structural differences related to regulation. The polarity of the environment near the N terminus of S1 was unaltered, but that of HMM was appreciably altered by nucleotide binding or by phosphorylation.

In the presence of nucleotides, the pyrene and ACR in UP-HMM are in a more polar environment than in P-HMM. This is the opposite from what might be expected from the addition of the phosphate group and suggests that the fluorophores are not influenced by the phosphate electrostatic environment. The more polar environment of UP-HMM cannot be explained by greater solvent accessibility, given the acrylamide quenching data, which suggests lower accessibility for pyrene. Therefore, this is most likely caused by a reduction in nonpolar interactions or closer interaction of charged groups with pyrene.

In the absence of nucleotides phosphorylation of pyrene-labeled A23C/RLC HMM decreased the emission intensity, indicating an increased polarity of the environment. This could be the result of greater solvent accessibility. However, the acrylamide-quenching data suggest that the accessibility is the same between the two states. Closer interactions with charged groups (possibly the P) or a reduction in existing interactions with nonpolar groups may also explain these data. The pyrene spectra for HMM, unlike the pyrene acrylamide quenching or the ACR-labeled HMM spectra, showed differences between the UP and P-HMM even in the absence of nucleotides. This is in agreement with our cross-linking results (22, 23), which showed that structural differences can be detected even in the absence of the motor domain.

In the absence of nucleotides, the emission spectra of ACR-labeled A23C/RLC HMM, in contrast to pyrene spectra but in agreement with pyrene quenching, was unaffected by phosphorylation. ATP and ADP significantly decreased the emission intensity of UP-HMM in an almost identical manner (Fig. 6, Table III), although they had relatively small effects on the spectra of P-HMM. Both nucleotides also caused an 8-nm red shift in the \( \lambda_{\text{max}} \) of ACR in UP-HMM, whereas they remained unchanged in P-HMM. These changes are in agreement with pyrene spectra, which indicated a net increase in polarity about the pyrene moiety upon nucleotide binding to UP-HMM. The lack of response to nucleotide binding in ACR-labeled P-HMM in contrast to pyrene-labeled P-HMM may be due to the smaller size of ACR, which may make it less sensitive to global changes. Overall, these data show that A23C/RLC of UP-HMM is in a more polar environment than that of P-HMM but only if nucleotide is present. Neither phosphorylation nor nucleotides had any significant effect on ACR-A23C/RLC S1. Once again this points to a regulation mechanism involving S1-S1 or S1-S2 interactions.

The N Termini of S1 and P-HMM RLC Are Not in Identical Structural States—Because of the difference in RLC exchange efficiencies, we were not able to quantitatively compare ACR fluorescence intensities between S1 and HMM (Table III). However, the red-shifted \( \lambda_{\text{max}} \) of ACR illustrates that the RLC N terminus of S1 is in a more polar environment than P-HMM. Also, the thiol reactivity rate of P-HMM is slower than P-S1, and the solvent exposure of P-HMM is less than P-S1, consistent with the notion that some degree of S1-S1 or S1-S2 interaction may occur even in P-HMM. Therefore, although P-HMM and S1 are more similar to one another than UP-HMM and S1, suggesting that the heads of P-HMM may act independently, our studies do detect significant differences between P-HMM

**Fig. 8.** Transients of a single turnover of FTP and ACR fluorescence for ACR-labeled A23C/RLC UP and P-HMM. The plots show the changes in the fluorescence intensities of FTP (left y axis) and ACR attached to residue 23 of RLC (right y axis). Two solutions, one containing the ACR-HMM and the other, FTP, were mixed in 1:1 ratio in a stopped-flow fluorometer, giving 1 \( \mu \text{M} \) final concentrations of both HMM heads and FTP. The transients were recorded using either formycin or ACR excitation and emission settings. A, UP-HMM. B, P-HMM. C, UP-HMM as in A, except data were collected for 2000 s, allowing complete turnover of FTP. The single-exponential fits to the nucleotide release phase (dotted lines) for the ACR and FTP were 0.0020 and 0.0014 (fit, S.E. <0.0001), respectively. These values are consistent with Table I.
and S1. This is consistent with steady-state ATPase activity data showing that S1 and P-HMM do not have the same level of activation; P-HMM is more active than S1 (7, 54–56). Most constructs that have altered regulation have not only an increased ATPase activity in the UP state but also a decreased ATPase activity in the P state. This suggests that the P-state does not reflect a simple case in which an inhibitory mechanism has been removed but may reflect a rapid equilibrium between on and off structural states, where the equilibrium is modified by the mutation. The findings point to a cooperative activation and deactivation mechanism that requires communication between the two heads. Therefore, selective interactions about the RLC in the UP or P states must modulate the type of head-head communication.

**Transient Studies Show a Direct Correlation between the Nucleotide Binding to the Catalytic Site and the Conformational Change in the RLC N Terminus of UP-HMM**—We have demonstrated that RLC N-terminal conformational changes reported by ACR were correlated in time to nucleotide binding and release (Fig. 7 and 8). The ACR signal amplitude was proportional to the occupancy of the catalytic sites with ATP (or ADP + Pi). Transients of the complete single turnover of the FTP in UP-HMM indicated that the observed conformational changes were reversible (Fig. 8). The studies also confirmed that such changes only occurred in the UP state of HMM. Although, FTP bound to ACR-labeled P-HMM, it did not change ACR fluorescence.

**Changes in ACR Fluorescence Signal May Report the Heads-up (Heads Extended away from Tail) to Flexed (Heads Folded Back toward the Tail) Transition in HMM**—The phosphorylation-dependent conformational changes we detected for the N terminus were most evident in the presence of nucleotides. Under these conditions UP-HMM has a higher sedimentation coefficient (57), a lower proteolytic susceptibility of the head-rod junction (58), a less mobile motor domain and RLC (59), and a lower basal ATPase activity (57) than P-HMM. UP-HMM appears to have heads flexed back toward the rod (60, 61), and P-HMM appears to have the heads extended away from the rods (60). It is known that the flexed and extended conformations of HMM are in rapid equilibrium for both smooth (57) and scallop isoforms (62, 63). The equivalent of these conformations for full-length myosin under physiological conditions is the folded 10 S, if in UP-SMM or calcium-deficient scallop myosin at low ionic strength, and the extended 6 S, if in P-SMM or calcium-bound scallop myosin. For both HMM and myosin the flexed conformation is stabilized in the presence of ATP or ADP.

UP-HMM in the flexed conformation can be transitioned to the extended conformation by the addition of salt within the range of about 0.2–0.4 M (57). We observed the nucleotide-induced ACR fluorescence change under conditions of relatively low ionic strength (50 mM NaCl; Table III and Figs. 5–7), but this signal change was abolished within an ionic strength range of 0.2–0.4 M NaCl (data not shown). Therefore, at high ionic strength, nucleotide does not affect a signal change for UP-HMM nor does it for P-HMM at low ionic strength (Table III) even though nucleotide binds under these conditions. This strongly suggests that a high ACR signal reports the heads-up conformation, whereas a low ACR signal reports the flexed conformation. Combining the information from acrylamide quenching and the spectral analysis, it appears that flexing of the heads places the RLC N terminus in a less exposed and more polar environment. This positioning of the N terminus is important to the stability of the flexed form, because it has been shown that removal of a portion of the N terminus can destabilize the 10 S (flexed in full-length SMM) conformation (21) even in the presence of nucleotides.

The fact that the heads-down or off state is stabilized in the presence of nucleotides for scallop and smooth HMM (57, 63) further supports the idea that our signal reports the heads-down conformation. Therefore, the difference we see in ACR signal between UP- (low) and P-HMM (high) is only observed in the presence of nucleotides, whereas in the absence of nucleotides the signals are identical and high. This suggests that the equilibrium between the two states is mostly toward the on state in the absence of nucleotides. This is in contrast with the acrylamide quenching data, which suggested that the two states were in approximately in equal amounts in the absence of nucleotides. Therefore, it seems that different attached probes give qualitatively the same conclusion; that there are at least two states that are in equilibrium but give different quantitative measures of the position of the equilibrium in the nucleotide-free state. This is likely due to the different structures of the two attached probes.

In summary, we report novel sensors of nucleotide binding and phosphorylation located at the RLC N terminus. The sensors are sensitive to these parameters if within the environment of a regulated subfragment of SMM with two heads but are insensitive within the environment of an unregulated subfragment with only one head. This is the first report of a fluorescence signal for nucleotide binding on any of the myosin light chains. Our data suggest that the sensor can monitor the transition from a flexed to extended conformation in HMM. This work opens up a new technique to understand structural changes in regulated myosin subfragments and will be a useful new tool to measure phosphorylation kinetics.

**Acknowledgments**—We thank Lilly Ng, Olivia Henderson-Hall, Ivan B. Anderson, Michelle Bogoger, Regina Barr, and Professor William Welch for assistance.

**REFERENCES**

1. Sellers, J. R. (1999) *Myosin*, 2nd Ed., Oxford University Press, Oxford
2. Sellers, J. R. (1991) *Curr. Opin. Cell Biol.* 3, 98–104
3. Rayment, I., Rypniewski, W. R., Schmidt-Base, K., Smith, R., Tomchick, D. R., Benning, M. M., Winkelman, D. A., Wesenberg, G., and Holden, H. M. (1993) *Science* 261, 59–58
4. Trybus, K. M., and Warshaw, D. M. (1991) *J. Cell Sci. Suppl.* 14, 87–89
5. Trybus, K. M., and Chatman, T. A. (1993) *J. Biol. Chem.* 268, 15142–15151
6. Benson, P. A., Sellers, J. R., and Cremona, C. R. (2000) *J. Biol. Chem.* 275, 15142–15151
7. Ikebe, M., and Hartshorne, D. J. (1985) *Biochemistry* 24, 2380–2387
8. Saita, M., Matsuura, M., and Ikebe, M. (1996) *Biochemistry* 35, 11113–11118
9. Matsu-ura, M., and Ikebe, M. (1995) *PEBS Lett.* 363, 245–250
10. Trybus, K. M. (1994) *J. Biol. Chem.* 269, 20819–20822
11. Konishi, K., Kojima, S., Katoh, K., Fujawa, M., Kato, K., Fujwara, K., and Onishi, H. (2001) *J Biochem.* (Tokyo) 129, 365–372
12. Sellers, J. R., Pato, M. D., and Adelstein, R. S. (1981) *J. Biol. Chem.* 256, 13137–13142
13. Cremo, C. R., Sellers, J. R., and Facemyer, K. C. (1995) *J. Biol. Chem.* 270, 2171–2175
14. Konishi, K., Kato, T., Morita, F., and Yazawa, M. (1996) *J. Biochem.* (Tokyo) 124, 163–170
15. Cremo, C. R., Wang, F., Facemyer, K., and Sellers, J. R. (2001) *J. Biol. Chem.* 276, 41465–41472
16. Trybus, K. M., and Lowey, S. (1988) *J. Biol. Chem.* 263, 16485–16492
17. Kato, T., and Morita, F. (1990) *J. Biol. Chem.* 271, 9992–9996
18. Ikebe, M., Reardon, S., Mitani, Y., Kamisoyama, H., Matsuura, M., and Ikebe, R. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 9096–9100
19. Trybus, K. M., and Chatman, T. A. (1995) *J. Biol. Chem.* 269, 4412–4419
20. Ikebe, M., and Morita, J. (1991) *J. Biol. Chem.* 266, 21339–21342
21. Ikebe, M., Ikebe, R., Kamisoyama, H., Reardon, S., Schwenk, J. P., Sanders, C. R. II, and Matsuura, M. (1994) *J. Biol. Chem.* 269, 28173–28180
22. Wu, X., Clack, B. A., Zhi, G., Stull, J. T., and Cremona, C. R. (1999) *J. Biol. Chem.* 274, 20328–20335
23. Wahlstrom, J. L., Randall, M. A., Jr., Lawson, J. D., Lyons, D. E., Siems, W. F., Crouch, G. J., Barr, R., Facemyer, K. C., and Cremona, C. R. (2003) *J. Biol. Chem.* 278, 5125–5131
24. Ichihara, M., Muras, K., Harada, F., and Nishimura, S. (1986) *Biochem. Biophys. Acts* 155, 82–90
25. Ikebe, M., and Hartshorne, D. J. (1985) *J. Biol. Chem.* 260, 13146–13153
26. Konishi, K., and Kojima, S. (1971) *J. Biol. Chem.* 246, 4866–4871
27. Adelstein, R. S., and Klee, C. B. (1982) *Methods Enzymol.* 85, 298–308
28. Hermanson, G. T. (1996) *Bioconjugate Techniques*, pp. 88–90, Academic Press, Inc., San Diego
29. Haugland, R. P., Spence, M. T. Z., and Johnson, I. D. (1996) *Handbook of Fluorescent Probes and Research Chemicals*, 6th Ed., Molecular Probes, Eugene, OR
30. Olney, J. J., Sellers, J. R., and Cremo, C. R. (1996) J. Biol. Chem. 271, 20375–20384
31. Facemyer, K. C., and Cremo, C. R. (1992) Bioconjugate Chem. 3, 408–413
32. De La Cruz, E. M., Sweeney, H. L., and Ostap, E. M. (2000) Biophys. J. 79, 1524–1529
33. Yang, K., Farrens, D. L., Hubbell, W. L., and Khorana, H. G. (1996) Biochemistry 35, 12464–12469
34. Watts, S. D., and Capaldi, R. A. (1997) J. Biol. Chem. 272, 15065–15068
35. Bass, R. B., and Falke, J. J. (1998) J. Biol. Chem. 273, 25006–25014
36. Walker, J. M. (1996) The Protein Protocols Handbook, Humana Press Inc., Totowa, NJ
37. Espenson, J. H. (1995) Chemical Kinetics and Reaction Mechanisms, 2nd Ed., McGraw-Hill Inc., New York
38. Ellison, P. A., DePew, Z. S., and Cremo, C. R. (2003) J. Biol. Chem. 278, 4410–4415
39. Britto, P. J., Knipling, L., and Wolff, J. (2002) J. Biol. Chem. 277, 29018–29027
40. Zhi, G., Herring, B. P., and Stull, J. T. (1994) J. Biol. Chem. 269, 24723–24727
41. Ikebe, M., Reardon, S., Schwonek, J. P., Sanders, C. R., II, and Ikebe, R. (1994) J. Biol. Chem. 269, 28165–28172
42. Wright, P. E., and Dyson, H. J. (1999) J. Mol. Biol. 290, 321–331
43. Dunker, A. K., Brown, C. J., Lawson, J. D., Iakoucheva, L. M., and Obradovic, Z. (2002) Biochemistry 41, 6573–6582
44. Kojima, S., Fujimura, K., and Onishi, H. (1999) Biochemistry 38, 11670–11676
45. Domínguez, R., Freyzo, Y., Trybus, K. M., and Cohen, C. (1998) Cell 94, 559–571
46. Trybus, K. M. (1994) J. Muscle Res. Cell Motil. 15, 587–594
47. Lakowicz, J. R. (1999) Principles of Fluorescence Spectroscopy, 2nd Ed., Kluwer Academic/Plenum Press, New York
48. Bartegi, A., Roustan, C., Kassab, R., and Fattoum, A. (1999) Eur. J. Biochem. 262, 335–341
49. Shi, J., Radic, Z., and Taylor, P. (2002) J. Biol. Chem. 277, 43301–43308
50. Cremo, C. R., and Geeves, M. A. (1998) Biochemistry 37, 1969–1978
51. Nyitrai, M., Szent-Gyorgyi, A. G., and Geeves, M. A. (2002) Biochem. J. 365, 19–30
52. Kalabokis, V. N., and Szent-Gyorgyi, A. G. (1997) Biochemistry 36, 15834–15840
53. Chandler, P. D., Sellers, J. R., and Szent-Gyorgyi, A. G. (1981) Biochemistry 20, 210–216
54. Sellers, J. R., Eisenberg, E., and Adelstein, R. S. (1982) J. Biol. Chem. 257, 12880–12883
55. Greene, L. E., Sellers, J. R., Eisenberg, E., and Adelstein, R. S. (1983) Biochemistry 22, 530–535
56. Onishi, H., Maeda, K., Maeda, Y., Inoue, A., and Fujiwara, K. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 704–708
57. Suzuki, H., Stafford, W. F., III, Slayter, H. S., and Seidel, J. C. (1985) J. Biol. Chem. 260, 14810–14817
58. Ikebe, M., and Hartshorne, D. J. (1984) J. Biol. Chem. 259, 11639–11642
59. Rosenfeld, S. S., Xing, J., Cheung, H. C., Brown, F., Kar, S., and Sweeney, H. L. (1998) J. Biol. Chem. 273, 28682–28690
60. Wendt, T., Taylor, D., Trybus, K. M., and Taylor, K. A. (1999) J. Cell Biol. 147, 1385–1390
61. Wendt, T., Taylor, D., Trybus, K. M., and Taylor, K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 4361–4366
62. Frado, L. Y., and Craig, R. (1992) J. Muscle Res. Cell Motil. 13, 436–446
63. Stafford, W. F., Jacobsen, M. P., Woodhead, J., Craig, R., O’Neill-Hennessey, E., and Szent-Gyorgyi, A. G. (2001) J. Mol. Biol. 317, 137–147
64. Garnier, J., Giblat, J. F., and Robson, B. (1998) Methods Enzymol. 266, 540–553

Smooth Muscle Myosin Regulatory Light Chain Structure
