The N-terminal Lobes of Both Regulatory Light Chains Interact with the Tail Domain in the 10 S-inhibited Conformation of Smooth Muscle Myosin*

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In the presence of ATP, unphosphorylated smooth muscle myosin can form a catalytically inactive monomer that sediments at 10 Svedbergs (10 S). The tail of 10 S bends into thirds and interacts with the regulatory domain. ADP-Pi is “trapped” at the active site, and consequently the ATPase activity is extremely low. We are interested in the structural basis for maintenance of this off state. Our prior photocross-linking work with 10 S showed that tail residues 1554–1583 are proximal to position 108 in the C-terminal lobe of one of the two regulatory light chains (Olney, J. J., Sellers, J. R., and Cremo, C. R. (1996) J. Biol. Chem. 271, 20375–20384). These data suggested that the tail interacts with only one of the two regulatory light chains. Here we present data, using a photocross-linker on position 59 on the N-terminal lobe of the regulatory light chain (RLC), demonstrating that both regulatory light chains of a single molecule can cross-link to the light meromyosin portion of the tail. Mass spectrometric data show four specific cross-linked regions spanning residues 1428–1571 in the light meromyosin portion of the tail, consistent with cross-linking two RLC to one light meromyosin. In addition, we find that position 59 can cross-link internally to residues 42–45 within the same RLC subunit. The internal cross-link only forms in 10 S and not in unphosphorylated heavy meromyosin (lacking the light meromyosin), suggesting a structural rearrangement within the RLC attributed to the interaction of the tail with the head.

Myosin II is a hexameric motor protein that consists of two HCs,2 two RLCs, and two ELCs. Each of the two head domains

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2 The abbreviations used are: HC, heavy chain; LC, light chain; RLC, regulatory light chain; ELC, essential light chain; SMM, smooth muscle myosin; HMM, heavy meromyosin; LMM, light meromyosin; BP1A, benzophenone-4-iodoacetamide; BP-S59C RLC, recombinant RLC with an Ala substituted at position 108 and a BP1A-labeled Cys substituted at position 59; Glu-C, endoproteinase Glu-C; MS, mass spectrometry; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; LC-ESI, liquid chromatography-electrospray ionization; MS/MS, tandem mass spectrometry; ASAP, Automatic Spectrum Assignment Program (52); CPK, Corey-Pauling-Koltun; MOPS, 4-morpholinopropanesulfonic acid.
closely spaced potential bends characterized by their low propensity for coiled-coil structure (31).

The tail-regulatory domain interaction is consistent with the fact that both the ELC and the RLC, and specifically the RLC N terminus are required for 10 S stability (32, 33). Single-headed SMM can fold, but only at an unusually low ionic strength (31). Therefore, the presence of both heads appears to strongly stabilize the interaction of the tail. Because both motor domains are required to adopt the off state (34), it appears the tail binds to the heads in the off state (31). This is consistent with mutagenesis data showing that myosin hybrids that cannot adopt the off state are less stable in the 10 S conformation (32).

The ATPase activity of the 10 S monomer is efficiently switched off. The products of ATP hydrolysis, ADP-Pi, are stably bound (trapped) at both of the 10 S active sites. Efficient active site trapping appears to be a unique property of 10 S. Neither 6 S monomers, in which the tail is extended, nor filaments can trap ADP-Pi, to such an extent. Measurements of product release rates have shown that 6 S extended SMM turns over at ~0.02 s⁻¹ and filaments turn over at 0.006 s⁻¹, whereas 10 S folded SMM turns over at ~2–4 x 10⁻⁴ s⁻¹ (35–37). 10 S must transition back to the 6 S conformation before its bound ADP-Pi can be released. An important question is whether or not the tail interaction with the head is required for the trapped state. Covalent attachment of the tail to the C-lobe of the RLC is sufficient to keep that head’s ATPase activity inhibited even under high ionic strength conditions, which activated the partner head. However, other significant interactions may also have been stabilized. Here we attached a photocross-linker to the other RLC lobe (N-terminal) at SS9C to determine whether the tail interacts with this lobe. After photo-activated cross-linking we find four types of molecules all of which are “locked” into the 10 S conformation: Molecule I in which both RLCs are cross-linked to one HC; Molecule II in which each RLC is cross-linked to an HC; Molecule III in which one RLC cross-linked to an HC and the other RLC remains uncross-linked; and Molecule IV in which one RLC is cross-linked to an HC and the other is internally cross-linked. Analysis of the sites of cross-linking by MALDI-MS confirmed that there are at least two regions of the tail, spaced ~117 Å, that interact with SS9C in 10 S SMM. Therefore, these data show that the tail interacts with both RLC at SS9C within a single molecule.

MATERIALS AND METHODS

Protein Preparations—SMM was prepared from frozen chicken gizzards (49) obtained from Pell-Freeze. All experiments were performed with 100% unphosphorylated SMM. Human smooth muscle RLC (MLRN_HUMAN, accession P24844) mutants were expressed and purified from Escherichia coli BL21(DE3) (34). Native ELC was obtained from the supernatant of a 95% ethanol precipitation of purified SMM (50). Protein concentrations were determined using the following extinction coefficients: SMM, ε₂₈₀ = 0.56; RLC, ε₁₇₇ = 0.337; BP-RLC, ε₀₂ = 0.89 (31); and ELC, ε₂₈₀ = 0.31.

Cross-linking SMM into 10 S—Sample preparation and cross-linking methods were as previously reported (31) and as in the figure legends. Briefly, recombinant thrombin-treated SS9C RLC was labeled with BPIA (synthesized as described (31)), exchanged onto SMM using trifluoroperazine (51) or heat (31), and the non-filamentous protein was irradiated in 10 S buffer conditions (31). After centrifugation in a filament-promoting buffer to remove filaments, the protein in the supernatant was applied to a gel-filtration column. The protein eluting at the 10 S position under high salt conditions was collected (see Fig. 1B).

Determination of the Composition of Gel-filtration Fractions—The structures and the approximate relative percentages of all the cross-linked molecules present in each fraction across the gel-filtration peak (Fig. 1B) were determined in five steps. First, gel band assignments were made using Rp values calculated from a molecular weight standard curve (see Blue Plus 2 (Invitrogen) plus the SMM HC). Second, stain densities were quantified (Lab Works software, Ultraviolet Products), and the values were normalized to the SMM standard (lane 1, Fig. 1A) to account for varying sample loads. All stain densities were verified to be in the linear range. Lane 2, Fig. 1B, was not ana-
lyzed due to the distortion of bands in the HC region. Third, we verified that the density lost from the RLC bands equaled the amount gained in the HC region, thus accounting for all material. Fourth, for the HC-RLC and RLC-HC-RLC bands, the fraction of each measured band density ($D_{band}$) that was attributed to RLC staining alone ($X$) was determined by Equations 1 and 2, respectively, where $[D_{RLC}/D_{HC}]_\text{std} = 0.16 = \text{band density ratio for unmodified SMM}$.

$$X_{RLC-HC} = D_{RLC-HC} \times 0.14 \quad \text{(Eq. 1)}$$

$$X_{RLC-HC-RLC} = D_{RLC-HC} \times 0.24 \quad \text{(Eq. 2)}$$

$$X_{RLC} = D_{RLC} \quad \text{(Eq. 3)}$$

$$X_{RL-RLC} = D_{RL-RLC} \quad \text{(Eq. 4)}$$

$$Y_{band} = \frac{X_{band}}{\sum X_{band}} \times 100 \quad \text{(Eq. 5)}$$

This adjustment was not necessary for the uncross-linked and internally cross-linked RLC bands, because they do not contain HCs; so $X = D_{band}$; see Equations 3 and 4. Fifth, the $X$ values for the four RLC-containing bands were converted to percentages $Y$ using Equation 5. Therefore, $Y$ represents the percentage distribution of the RLC among the four RLC containing bands. These values were used to estimate the relative amounts of the four cross-linked molecules present in each fraction as described under “Results” for Fig. 3.

**Proteolytic Digest of Photocross-linked 10 S—**High-performance liquid chromatography-purified cross-linked 10 S SMM or unmodified SMM (1.5 mg/ml in 10 mM sodium phosphate, 1 mM MgCl$_2$, 0.1 mM EGTA, 125 mM NaCl, 0.5 mM dithiothreitol, 2 mM ATP, pH 7.5) were equilibrated (25 °C with stirring) prior to an additional wash. Liquid was removed and 50 μl of acetonitrile was added for 5 min at room temperature prior to an additional wash. Liquid was removed and the RLC digest (25 μl, 10 μg/ml in ammonium bicarbonate, pH 8.5) was performed at 37 °C for 16 h. The reaction was quenched with 15 μl of 0.3% formic acid. Peptides were extracted from the gel material as described above.

**Mass Spectral Analysis—**Samples (pH adjusted to <4) were aspirated and dispensed with ZipTips (ZipTipI-C18 PB TMP, Millipore) three times, and the bound peptides were eluted with 70% acetonitrile/0.2% formic acid containing 5 mg/ml α-cyano-4-hydroxycinnamic acid matrix (Aldrich), then spotted onto the MALDI target. Mass spectra were obtained using an Applied Biosystems 4700 Proteomics Analyzer with TOF/TOF optics. The samples were run in positive reflector mode at laser power 5,100 or 2,500 scanning from 700 to 4,000 Da and accumulating 10,000 shots/spectra. Three internal standards (Sigma) were applied to the samples; angiotensin I, m/z 1296.68; adrenocorticotropic hormone fragments (clip 1–17), m/z 2093.087; and adrenocorticotropic hormone (clip 7–38), m/z 3657.929. Tandem MS spectra were recorded in positive-ion mode with a resolution of 50 (full-width at one-half maximum) and 2500 shots were collected. For collision-induced dissociation-MS/MS, the laser power was 5500, and the selected ions were fragmented using atmospheric gas in the collision cell and analyzed in the orthogonal TOF. Some spectra were taken without the gas, termed laser-induced dissociation-MS/MS at laser power 6500.

**Analysis of MALDI-TOF/TOF Data to Determine the Sites of Cross-linking—**Precursor mass lists with signal to noise levels ≥ 20 were generated from MALDI-TOF/TOF experiments. To annotate the mass lists, data were submitted on-line for analysis by ASAP (Sandia Laboratories) (52). For each experiment, the appropriate criteria were entered as variables; tolerated error value was ± 25 ppm, cross-linker mass was 237.086 Da, and methionine oxidation and protease specificity were used. For the RLC-LMM cross-link, the LMM sequence (MYH11_CHICK, accession P10587, residues 1150–1979) and the RLC sequence (MLRN_HUMAN, accession P24844 with N terminus extended with the sequence GSHM and Ser-59 mutated to Cys and Cys-108 mutated to Ala) were entered with an intervening nonsense KKKKK sequence. For the internal RLC cross-link, the RLC sequence was entered. The benzophenone modification was restricted to S59C but was permitted to photo-insert into any residue either with or without the loss of water (48). The ASAP output is an annotated list of possible assignments for all mass lists, including unmodified peptides and possible cross-linked peptides. The same procedure was repeated for the precursor mass list obtained from a gel blank sample and for a benzophenone-labeled but unirradiated S59C sample (or the unirradiated LMM for the LMM-RLC data). The mass lists, calculated for protease autolysis (determined by MassCutter, us.expasy.org) and human keratin con-

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**Proteolytic Digest of Internal RLC Cross-links—**The internal RLC cross-link (Fig. 1B), unirradiated BP-S59C, native RLC, and blank gel bands were excised from the gel and placed into microcentrifuge tubes. Gel plugs were destained in 50% acetonitrile, 100 mM ammonium bicarbonate. Liquid was removed, and 50 μl of acetonitrile was added for 5 min at room temperature prior to an additional wash. Liquid was removed and the RLC digest (25 μl, 10 μg/ml in ammonium bicarbonate, pH 8.5) was performed at 37 °C for 16 h. The reaction was quenched with 15 μl of 0.3% formic acid. Peptides were extracted from the gel material as described above.

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RESULTS

Preparation and Characterization of SS9C RLC SMM—The RLCs and ELCs were removed from native SMM (Fig. 1A, lane 1) using trifluoperazine (lane 2) (51) and replenished with native ELCs and BP-SS9C RLCs (lane 3). Some samples were prepared by a heat-exchange procedure (31). We have previously shown that BP-SS9C RLC HMM has normal phosphorylation-dependent regulatory properties as measured by steady-state actin-activated ATPase activity (34). Single-turnover rates (38) at 12.5 and 25 μM actin were 0.0017 and 0.0012 s⁻¹, respectively. Control values were 0.0005 ± 0.0005 s⁻¹. Therefore this preparation had a slightly elevated ATPase rate consistent with the study below.

The solubility of SS9C RLC SMM was assessed by determining the percentage of soluble versus filamentous myosin (1 mg/ml) at various ionic strengths (Fig. 2) in the presence of Mg-ATP. The control SMM was 100% soluble (did not form filaments) throughout the range of ionic strengths, which was as expected from previous work (56). At lower ionic strengths the equilibrium between 10 S and 6 S is shifted toward 10 S,

Structure of 10 S Smooth Muscle Myosin by Photocross-linking

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whereas at the higher ionic strengths 6 S is favored (57). The mutant SMM was completely soluble at higher ionic strengths indicating that the sample did not contain irreversibly aggregated material, which might have given erroneous cross-linking results. At the lower ionic strengths the mutant SMM was less soluble than control SMM. This indicates that the mutant SMM is not as stable in the 10 S conformation as the control. Approximately 52% of the mutant SMM was soluble at 125 mM NaCl, which is the salt concentration of the irradiation buffer (see below). Therefore, the incorporation of S59C RLCs lowered the critical monomer concentration (concentration at which filaments assemble). However, we obtained sufficient soluble mutant SMM for structural studies after removing the residual filaments by centrifugation.

**Preparation and Characterization of Photocross-linked 10 S SMM—Irradiation of BP-S59C SMM (filaments removed) under 10 S conditions (Fig. 1A, lane 4), resulted in two covalent cross-links between BP-S59C RLC and the HC (RLC-HC and RLC-HC-RLC) and an internal RLC cross-link (XL RLC). Centrifugation of this preparation in a filament-promoting buffer (10 mM MOPS, pH 7.0, 0.1 mM EGTA, 50 mM NaCl, 1 mM dithiothreitol, 5 mM MgCl₂) gave a supernatant that was enriched in cross-linked SMM, which could not form filaments, and a pellet (filaments) enriched in uncross-linked SMM (data not shown). The conformation of the SMM in the supernatant was analyzed by gel filtration under high ionic strength (6 S) conditions (Fig. 1B). The elution volume of the predominant peak was 14.55 ml. In contrast, unmodified SMM, which is in the 6 S conformation at this ionic strength, eluted at 12.2 ml. In contrast, the cross-linked SMM was unable to adopt the 6 S conformation under these high ionic strength conditions. These data suggest that the cross-linked SMM was "locked" in the 10 S conformation and is unable to adopt the 6 S conformation under these high ionic strength conditions. These data suggest that the cross-linked SMM adopts the "native" 10 S conformation, as measured by gel filtration.

To determine the molecular species present in the gel-filtration peak, protein from each collected fraction was analyzed by SDS-PAGE (Fig. 1B). All three cross-linked bands were present across the peak. For each lane, the fraction of the band density that could be attributed to RLC staining alone was calculated for all bands containing RLC (X value, see "Materials and Methods"). X values were then converted to Y values, which represent the percentage distribution of the RLCs among the 4 RLC-containing bands in that lane. For the representative gel lane in Fig. 3, the cross-linked RLC band represented ~15% of all the RLCs (Fig. 3A). This defined one half of Molecule III (Fig. 3C) to contain an uncross-linked RLC at ~15% plus an HC-RLC at ~15%. Pairing with an HC-RLC (½ molecule) was necessary to account for the fact that the complete Molecule III was locked in the 10 S conformation, thus explaining the behavior by gel filtration. The complete Molecule III formed by this assignment accounted for ~30% of all complete molecules in the peak (SMM %, Fig. 3C). A similar calculation can be done for the internally cross-linked band RLC in relation to Molecule IV.

The RLC-HC-RLC band representing ~41% of all RLCs (Fig. 3A) showed that Molecule I was ~28% of all complete molecules in the fraction (Fig. 3C). Finally, the HC-RLC band (Fig. 3A) representing ~34% of all RLCs showed Molecule II to be ~9% of all complete molecules after considering the amount of the HC-RLC in Molecules III and IV shown above. Fig. 3B illustrates how the molecular compositions of Molecules I–IV in terms of RLC subunits (%) Fig. 3C add up to account for the Y values from the gel (Fig. 3A).

The molecular compositions of all gel-filtration fractions from Fig. 1B were approximately the same (data not shown). Each fraction contained the four different types of cross-linked molecules, defined in Fig. 3C. Molecule I has both RLCs cross-linked to the same HC subunit; Molecule II has both RLCs cross-linked to different HC subunits; Molecule III has one RLC cross-linked to a HC and the other RLC remains un-cross-linked; and Molecule IV has one RLC cross-linked to a HC and the other RLC is internally cross-linked. The presence of Molecules I and II in significant quantities suggests that the tail interacts with both RLCs in 10 S. Therefore there should be at least two RLC-interacting regions on the tail.

**FIGURE 3.** Assignment of molecular composition of cross-linked SMM in gel-filtration fraction 1 from Fig. 1B (lane 1). The identity and the corresponding Y value (see Equations 1–5) for each band that was used in the calculations are shown in A along with the gel scan from Fig. 1B (lane 1). The four SMM molecular structures, I–IV, present in the sample are shown in B depicting the RLC subunits (rectangular boxes) and cross-links (connecting lines) to the HC subunits (vertical bars). Note that each molecule contains at least one HC-RLC cross-link, because it eluted at the 10 S position on the gel-filtration column (locked in 10 S). Structures I–IV and their relative percentages (SMM %) account for the data from the gel scan. B shows the RLC subunit summation (%) adjacent to the appropriate band (A) that reconciles the molecular composition (C) with the gel data (A). The colored boxes and numbers assist in understanding how the RLC subunits were quantitatively accounted for to agree with the gel data. Molecule I has both RLC subunits cross-linked to one HC subunit and therefore is composed of one RLC-HC-RLC. Molecule I constitutes 41% of the total myosin present in the fraction (RLC subunit summation = 20.5 + 20.5 = 41%, in agreement with the gel data). Molecule II has one RLC cross-linked to one HC subunit and the second RLC cross-linked to the second HC subunit and therefore is composed of two HC-RLCs. Because both Molecules III and IV also contain HC-RLC, the total HC-RLC was distributed accordingly between Molecules I, II, and IV. The distribution is constrained by the % uncross-linked RLC (15%) that is present in Molecule III and the % internally cross-linked (XL) RLC (10%) that is present in Molecule IV. The sum of the HC-RLC subunits is 4.5 + 4.5 + 15 + 10 = 34%, and therefore is consistent with the gel data in A. The final composition of the fraction in % of total SMM molecules in C adds up to 100%. Note that this analysis is with respect to the RLC-containing subunits, and therefore the unmodified HC, although present on the gel, is already accounted for in Molecules I, III, and IV, and therefore need not be included in this calculation. An alternative calculation with respect to the HC was done (not shown), and the results were essentially the same. The ELC is not required for calculations by either method as it is not cross-linked.
Identification of HC-RLC Photocross-linked Regions—To identify the region of the HCs that cross-linked to S59C RLC, the fragments from a limited Glu-C digest were analyzed by SDS gel electrophoresis (Fig. 4). Fragments produced from a digest of unmodified SMM have been previously characterized (49). As can be seen in lane 1, the HMM/LMM junction is susceptible to cleavage (49, 58) leading to predominantly HMM and LMM, with a small amount of S1 and full-length tail. The two major bands unique to the cross-linked SMM digest (lane 6) were identified as RLC-LMM and RLC-LMM-RLC. The RLC-LMM-RLC band likely also contains some full-length tail, and therefore was not analyzed by MS (see below). Both bands cross-reacted with an anti-RLC antibody by Western analysis (data not shown). The involvement of LMM in the cross-link was further supported by the diminished S1/LMM ratio (compare lane 6 to control in lane 1). The absolute molecular weights were smaller than predicted due to a partially digested RLC, but the molecular weight shifts from LMM to LMM-RLC to RLC-LMM-RLC were as predicted.

To identify the LMM region involved in the cross-link, the LMM-RLC gel band was excised from four identical gel lanes and digested with both Glu-C and trypsin, and the fragments were submitted for MALDI-MS analysis. Table 1 shows the HC peptides found to be cross-linked to Ser-59 RLCs, and Fig. 5A shows their relative positions within LMM.

Identification of the Internal RLC Cross-link Position—The internally cross-linked RLC band (Fig. 1B) was excised and digested with Glu-C and analyzed by MALDI-TOF. The MS base peak spectra for the cross-linked, uncross-linked, and blank samples were analyzed as described under “Materials and Methods,” revealing a single peptide at m/z 2312.110 that was found only in the cross-linked sample. This m/z uniquely matched 53LHDM(ox)KGLKNPTDE66 cross-linked to 42QRND45 with an error of ±24.98 ppm (theoretical = m/z 2312.052). The signal to noise ratio of the precursor mass was low, just above our cut-off of 20 (21 actual), and not uncommonly for cross-linked peptides, a poor tandem MS spectrum was obtained (Table 2). Only peaks with signal to noise above 10 were considered for assignment, and all these peaks were assigned by MS2Assign. Even though we were able to prepare considerable quantities of digests, the characterization of the cross-link was difficult, because other enzyme and chemical digests did not produce any acceptable masses attributed to cross-linked peptides (data not shown). This suggests that the peptides were consistently poorly extracted from the gel and/or that they inefficiently desorbed from the MALDI plate. Further studies with an appropriate LC-ESI MS are required to fully characterize the internal RLC cross-link(s).

**DISCUSSION**

Characterization of S59C RLC SMM—We previously showed that S59C RLC HMM has normal phosphorylation-de-
FIGURE 5. In A: Top, ribbon rendering of a homology model of the SMM LMM region (residues 1200–1650) extrapolated from the coordinates of (53). The position of the cross-linked regions (labeled a–f) from Table 1 are in space-filling CPK rendering. The position of the S2/LMM junction (Bend one, residue 1175) and predicted Bends two and three from (27) are indicated. The area cross-linked to Cys-108 (27) is shown as an orange ribbon. Bottom, corresponding LMM region rendered with electrostatic potentials mapped to a van der Waals surface. Charge density plot was prepared in DelPhi (55). HC residue numbers are shown below.

B, schematic of the entire SMM molecule in the 10 S conformation. Starting from the regulatory domain, shaded connections indicate attachments to the S2 region (residue 850–1150) rendered as described in A. At the end of the S2 region is Bend one, which connects (not shown) to the region of LMM depicted in A (–1200–1650). Note that the tail regions are shown as linear and are not meant to depict the actual path of the tail. Positioning of the cross-linked regions nearby to the two SS9C residues (space filling) suggest that cross-linking is possible. The distance between the two SS9C residues is 177 Angstroms. This figure was prepared using Insight II (Accelrys).
pended regulation prior to UV irradiation (34), therefore, S59C RLC SMM is a good construct for a structural study of the off state in 10 S. Here we showed (Fig. 2) that the recombinant RLC significantly destabilized the 10 S conformation suggesting that the Ser-59 residue is important to 10 S stability. In light of normal kinetic properties described above, the mechanism for 10 S destabilization is likely through direct destabilization of RLC-tail interactions.

**Characterization of Irradiated BP-S59C RLC SMM**—After purification to remove uncross-linked material, the irradiated mutant SMM was analyzed by gel filtration to assess the molecular conformation. Nearly all of the material applied to the column eluted at the 10 S position even under high ionic strength conditions known to stabilize unmodified SMM in the 6 S conformation. Semiquantitative analysis of SDS gels of the column fractions (Fig. 1B) confirmed that all band densities could be explained by the presence of four differentially cross-linked molecules (Fig. 3) each of which contained at least one HC-RLC cross-link, thus explaining the elution properties. All molecules were “locked” in the 10 S conformation, because the tails were not able to extend to the 6 S conformation as would be normally observed for unmodified SMM under these chromatographic conditions. The fact that all four cross-linked molecules eluted at the 10 S position strongly suggests that they adopt the 10 S conformation.

The presence of molecules containing one RLC-HC-RLC (Molecule I) or two HC-RLC (Molecule II) shows that the tail interacts with the N-terminal lobes of both RLCs in 10 S SMM. It is logical to expect both types of cross-linked molecules if the coiled-coil presents each helix equally to each Ser-59 allowing the benzophenone to cross-link to either HC.

Limited proteolysis studies showed that the cross-linking was from the RLC to the LMM region of the HC (Fig. 4), which is the C-terminal two-thirds of the tail, as expected from our previous work (31). The proteolysis confirmed that both RLC cross-linked to LMM, as evidenced by the presence of an RLC-LMM-RLC band on the gel (Fig. 4). This finding strongly supports our interpretation of the composition of molecules found in the gel-filtration peak (Fig. 3).

An important question is whether or not the first cross-linking event forced a second cross-link that does not represent a *bona fide* interaction site. We think this is unlikely based upon our similar experiments with benzophenone on Cys-108 (31). Only a single Cys-108 RLC participated in cross-linking under identical irradiation conditions. Therefore, cross-linking of a second RLC to the HC is not an inevitable consequence of attaching the first cross-link.

**Analysis of Sites of LMM-RLC Cross-linking**—MALDI-TOF data were analyzed using ASAP (52), a program designed to process data generated from cross-linking experiments. The cross-linked peptides identified from the LMM-RLC digest (Table 1) revealed an interesting pattern of cross-linking within the N-terminal one-half of LMM (Fig. 5A). All cross-links fell into families, denoted a–f, containing overlapping or nearly overlapping LMM sequences. Peptide families are expected due to missed and differential proteolytic cleavages, methionine oxidation, protein dynamics, and the ability of benzophenone to react with any proximal residue or peptide backbone. No cross-links were found in the S2 region (see Fig. 5B) or in the C-terminal one-half of LMM (not shown in Fig. 5). Cross-links a–f are *bona fide* intramolecular cross-linked peptides, because they were isolated from molecules that migrated as monomers on gel filtration (Fig. 1B).

The mass spectral data do not provide information about the relative abundance of any particular cross-link family. However, we can infer that regions c, d, e, and f (Fig. 5A) represent the major sites of tail-RLC interaction in 10 S, because they are consistent with images of single 10 S molecules (3, 6, 9, 10, 29). Region f overlaps with our previously identified BP-C108–RLC 10 S SMM cross-linked region (residues 1554–1583, Fig. 5A, *orange*). The presence of multiple cross-linked regions supports our gel analysis (Figs. 1, 3, and 4) in that at least two positions within the LMM region of the tail interact with the two RLC within a single molecule. Regions c–f are near two predicted bends in the coiled-coil (31) as might be expected, because the coiled-coil is known to form a looped shape at the regulatory domain interaction region. Regions c–f overlap with relatively neutral to positively charged regions of the tail surface. This might be expected, because the RLC surface is predominately negatively charged.

Fig. 5B shows a schematic that explains why we might observed four regions of the coiled-coil involved in cross-linking (c–f). We know that at least two regions must be cross-linking within one molecule. The minimum linear distance (from the C terminus of d to the N terminus of f) is ~98 Å, and the maximum linear distance (from N terminus of d to the C terminus of f) is ~136 Å with a middle-to-middle distance of ~117 Å. This distance relationship is nearly identical between regions c and e. Interestingly, 117 Å is almost exactly the distance between the two Ser-59 along a reasonable curved path (not shown in Fig. 5B) following the surface of the regulatory domain in our regulatory domain model derived from photocross-linking data in unphosphorylated HMM (48). The
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Fig. 5B (left panel) depicts the two Ser-59 within the regulatory domain, the descending coiled-coil (850–1150), a break at Bend 1 representing the S2/LMM junction, and the ascending region 1200–1400 followed by the portion of the tail containing the cross-links c–f. Tail regions are shown as linear and do not represent the actual path of the tail. The continuation of the tail to the C terminus is not shown. Regions c and e are positioned proximally to the two Ser-59 residues.

Fig. 5B (right panel) shows a similar relationship for d and f. Note that the cross-linked region in the right image has been shifted 2 × 28 residues to the left causing the ascending region of the coiled-coil to move down. If antiparallel inter-coil charge-charge interactions stabilize 10 S, this model would account for maintaining the stability because of the 2 × 28 residue shift. The repeating unit of the coiled-coil spanning a positive and negative region is 28 residues (note that the relative positions of the ascending and descending coiled-coils are not known and are therefore not exact in Fig. 5). The transition between the c and e (left) to the d and f (right) positioning of the coiled-coil might also be explained by a shortening of the coiled-coil between Bends 1 and 2. Sheng et al. (59) showed that this tail region and only this tail region shortens by 4 nm upon phosphorylation of the RLC, as measured by atomic force microscopy. They also showed, as have others (28), that unphosphorylated SMM exists as a mixture of two conformations, distinguished by the attitude of the heads; down toward the tails favored in the unphosphorylated state versus extended up away from the tails favored in the phosphorylated state. It may be that our c and e cross-linking represents one of these conformations, and the d and f cross-linking represents the other. Alternatively, the heads-down to heads-up transition alone (not depicted in Fig. 5) without invoking tail shortening or visa versa could potentially explain the results.

These possible scenarios suggest that the RLC-tail interaction may be dynamic and/or able to adopt multiple conformations. Further studies will be required to firmly establish the mechanistic basis for the multiple cross-linking sites.

Note that the schematic in Fig. 5B, which only considers the relationship between the two Ser-59 residues and the tail, could also apply to the cryo-EM model, except that the two Ser-59 would be 18 Å apart. The data presented here concerning the spatial relationship between the two Ser-59 and the tail are consistent with any model that has the two Ser-59 < 117 Å apart.

The basis for cross-linked regions a and b (Fig. 5A) is puzzling, because these sites cannot be accounted for by the RLC-tail interaction discussed above, which requires a bend at the S2-LMM junction (Bend 1). Cross-links a and b require tail bending at positions N-terminal to the S2-LMM junction, and possibly further bending to adopt a compact shape consistent with the observed elution at the 10 S position by gel filtration. We considered the possibility that the cross-links could result from interactions between the two 10 S-shaped monomers in the 15 S dimer (27). This is unlikely, because the 15 S dimer is not stable in the buffer used during the photocross-linking nor would it migrate at the 10 S position on the gel-filtration col-umn. Zhang et al. (60) have shown that the SMM tail has a propensity to bend not only at the S2/LMM junction (Bend 1) and at Bends 2 and 3 (Fig. 5A) but at a site ~24 nm from the head-tail junction as well. A hairpin bending at this position could bring a close to Ser-59 on the RLC, depending upon the attitude of the heads. We have yet to reconcile cross-linking at region b with observed bending sites.

Position S59C of the RLC Can Form an Internal Cross-link in 10 S Myosin—We have previously investigated the spatial relationships between the two RLCs in unphosphorylated smooth HMM. Using the same benzophenone-labeled S59C RLC used here, we found that S59C did not cross-link internally or to any other subunit in either the unphosphorylated or phosphorylated HMM (34). LMM cross-linking was not expected, because HMM lacks LMM and therefore cannot form the intramolecular hairpin bend characteristic of 10 S. However, here we find that BP-S59C RLC can cross-link not only to the tail’s LMM region (Fig. 4) but also internally (Fig. 1). The presence of an internal RLC cross-link in 10 S and not in HMM suggests that the tail interaction with the regulatory domain is responsible for the difference. We propose that this detected structural difference in the RLC reflects the further stabilized off state, thus explaining the observed ~10-fold lower ATPase activity of 10 S SMM versus unphosphorylated HMM (36, 37).

A single internal S59C RLC cross-linking peptide with a mass consistent with [33]HDLMLACLGNPTD66 and [43]QNRD45 was observed after MALDI-MS analysis of several different protease and chemical digests. Unfortunately, due to a low signal and poor fragmentation, the tandem MS analysis (Table 2) was consistent with but did not conclusively confirm this assignment. We routinely observe poor fragmentation of cross-linked peptides using the MALDI-TOF/TOF under both collision-induced dissociation-MS/MS and laser-induced dissociation-MS/MS conditions (61).

Fig. 6 shows a portion of the RLC homology model from Wahlstrom et al. (48). Benzophenone (magenta) is attached to S59C (violet), which is a solvent-accessible residue (47) at the C-terminal end of helix B (62). The flanking residues Glu-42 (lime green) and Asp-45 (light blue) in space-filling CPK represent the internally cross-linked region 43QQNRD45. Although we have not been able to identify the exact cross-linked residue(s), in this conformation the reactive carboxyl of the benzophenone is within an Angstrom of several of the heteroatoms of Glu-42. A survey of the structural data base for RLC homologues, including calmodulins, showed that the helix A-loop-helix B region is structurally invariant. We suggest that tail interaction with this region in 10 S alters the position of the benzophenone to provide a more efficient cross-linking to [33]QNRD45 while still allowing cross-linking to the tail. The tail interaction may also be altering the RLC conformation elsewhere.

Previous work from this laboratory showed that the tail can also interact with Cys-108 in the C-terminal lobe of one RLC (31). Therefore at least one Cys-108 and one S59C are proximal in 10 S, and both can access region f of the tail (Fig. 5A). Because the Cys-108 and S59C within a single RLC are ~45 Å apart, the proximal Cys-108 and S59C in 10 S must be from different RLCs to account for the fact that they both can cross-link region f, assuming that the crystal structure is representative of the...
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RLC structure in the 10 S state. This fact places constraints on the relative positioning of the two RLCs with respect to each other in 10 S. The positioning of the C-terminal lobe near to the N-terminal lobe is consistent with our model of the regulatory domain (48). That model was derived partially from the fact that Cys-108 (C-terminal lobe) can cross-link in unphosphorylated HMM to position 71–81 in the C-terminal RLC lobe of the other head. In contrast, the data presented here, and the data supporting our regulatory domain model do not appear to be consistent with the structure modeled into the cryo-EM data of either HMM (41, 42) or 10 S (43). We do not know the reason for these inconsistencies other than that perhaps the solution structures that we are probing are different from the structure proposed from the two-dimensional crystal data. A strength of the photocross-linking approach is that it can capture a range of accessible conformations resulting from protein dynamics. At the same time it is always possible that a rare conformation is also captured. However, it is unlikely that our data, which is internally consistent from three different studies representing eight different cross-links, are all due to a rare conformation(s).

Other studies support our conclusion that the tail of 10 S SMM interacts with both RLCs. Rotary-shadowed (3, 27–30) and negative-stained (6) images strongly suggest that a conformation exists in which the tail is interacting with both heads. SMM hybrids containing skeletal RLC can form partially folded structures in which the tail bends at the S2/LMM junction to allow interaction with the regulatory domain, but the second bending region is not stabilized giving a partially folded structure (33). A hybrid with smooth muscle RLC in which the N terminus is truncated shows similar partially folded structures. However, a preparation of approximately one-half full-length smooth RLCs and one-half N-terminal truncated RLCs can form 10 S. Therefore, skeletal or smooth RLCs with truncated N termini are sufficient for the first interaction, whereas the second interaction to complete the folded structure requires the N terminus of smooth RLCs. These data are consistent with the idea that the native interaction of the tail may require two RLCs. From our data, we predict that region c or d participates in the first interaction and region e or f in the second.

In summary, our photocross-linking study provides new insights to the structure of 10 S myosin II from smooth muscle. It was conducted in solution, and therefore the results are likely to reflect 10 S solution conformation(s). By a thorough analysis of highly purified photocross-linked 10 S preparations, we have characterized four cross-linked 10 S structures. One of the structures contains one internally cross-linked RLC that is not found in unphosphorylated HMM. This structural difference may be attributed to the lower ATPase activity of 10 S versus HMM. For two other cross-linked structures, the N-terminal lobes of both RLCs in the region encompassing S59C simultaneously interacted with LMM. MS data showed that the cross-links within LMM are ~117 Å apart in an extended coiled-coil. These data along with our prior photocross-linking data are consistent with our regulatory domain model. Our present and prior data show that the tail of 10 S interacts with both RLC lobes. Previously it has been shown that cross-talk between the two RLC lobes is important to regulation of the ATPase (40). Therefore, our structural findings may be relevant to the mechanism of down-regulation of the SMM ATPase. Further studies are underway using multiple cross-linking approaches with the goal of modeling interactions of the tail with the entire SMM molecule.

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