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Phosphorylated smooth muscle heavy meromyosin shows an open conformation linked to activation

Bruce A. J. Baumann¹, Dianne W. Taylor¹, Zhong Huang¹, Florence Tama², Patricia M. Fagnant³, Kathleen M. Trybus³, and Kenneth A. Taylor^{1,§}

¹Institute of Molecular Biophysics, Florida State University, Tallahassee, FL 32306

²Department of Chemistry and Biochemistry, University of Arizona, Tucson, AZ 85721

³Department of Molecular Physiology and Biophysics, University of Vermont College of Medicine, Burlington, VT 05405

Abstract

Smooth muscle myosin and heavy meromyosin (smHMM) are activated by regulatory light chain (RLC) phosphorylation but the mechanism remains unclear. Dephosphorylated, inactive smHMM assumes a closed conformation with asymmetric intramolecular head-head interactions between motor domains. The “free head” can bind to actin, but the actin-binding interface of the “blocked head” is involved in interactions with the free head. We report here a 3-D structure for phosphorylated, active smHMM obtained using electron crystallography of 2-D arrays. Head-head interactions of phosphorylated smHMM resemble those found in the dephosphorylated state, but occur between different molecules, not within the same molecule. The light chain binding domain structure of phosphorylated smHMM differs markedly from that of the “blocked” head of dephosphorylated smHMM. We hypothesize that RLC phosphorylation opens the inhibited conformation primarily by its effect on the blocked head. Singly phosphorylated smHMM is not compatible with the closed conformation if the blocked head is phosphorylated. This concept has implications for the extent of myosin activation at low levels of phosphorylation in smooth muscle.

Keywords

Phosphorylation; 2-D crystalline arrays; myosin regulation; myosin light chains; lipid monolayers; electron crystallography

Introduction

Smooth muscle myosin (smM) is a class II myosin ¹ consisting of two heavy chains (HC) and two pairs of light chains, the essential light chain (ELC) and the regulatory light chain (RLC). The N-terminus of the myosin HC forms a globular motor domain (MD) that contains the ATP hydrolysis and actin binding regions. This is followed by a long, mostly α -helical segment to which the light chains bind, called the light chain domain (LCD) (Fig.

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[§]Corresponding author: taylor@bio.fsu.edu. .

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1B). The C-terminal halves of both HCs join together to form a long α -helical coiled-coil rod that is responsible both for forming the native dimer, through the S2 region of the rod, and for filament formation through the light meromyosin region. When the myosin head (MD plus LCD, also called S1) is bound to an actin filament, conformational changes within the MD are amplified by the LCD acting as a lever arm to produce filament sliding ².

The actin activated ATPase activity in smM is regulated by phosphorylation of ser19 on the RLC (reviewed in ³). Both full-length myosin and the two-headed subfragment, heavy meromyosin (smHMM), have low actin activated ATPase activity in the dephosphorylated state. Phosphorylation of the RLC increases the ATPase activity up to 1000-fold ⁴. However, neither the single headed fragment S1 ^{5; 6} nor single headed smHMM ^{7; 8} are regulated, but remain activated independent of the state of RLC phosphorylation. Smooth muscle regulation thus requires two heads, in addition to an intact S2 domain with a minimal length of 15 heptads (1 heptad = 7 sequential α -helical residues) ⁹, and phosphorylatable RLCs ¹⁰. The necessity for a 2-headed species implies that some form of head-head interaction is needed to achieve inhibition.

The first direct structural explanation for inhibition came from 2D arrays of phosphorylated and dephosphorylated smHMM ¹¹. 3D reconstructions of the dephosphorylated smHMM revealed an asymmetric structure (Fig. 1A) where the actin binding interface of one head, the “blocked” head, was bound to the companion head, the “free” head, on the same molecule ¹². Actin binding to the “blocked” head was inhibited, but the actin-binding interface of the free head was unobstructed. The 3- to 4-fold lower affinity of dephosphorylated versus phosphorylated smHMM for actin in the presence of MgATP could reflect the difference between single and double-headed binding ⁶. It was further proposed that the free head of dephosphorylated smHMM was inhibited because binding of the blocked head prevented converter domain motions that are required for fast phosphate release ¹². Thus both heads are inhibited, albeit by different mechanisms. Modeling of the 2D projection image of the phosphorylated smHMM could not be unambiguously interpreted, but pointed to an open or extended conformation ¹¹.

The inhibited state of full-length smM is soluble and incompatible with filament formation. This conformation has a sedimentation coefficient of 10S compared with a sedimentation coefficient of 6S for the extended, filament forming conformation, and is characterized by an asymmetric head-head interaction like that of dephosphorylated smHMM ¹³. The arrangement of molecules is completely different in the 10S myosin and smHMM 2-D arrays, strengthening the idea that intramolecular head-head interactions are an intrinsic property of the molecule, and not a product of crystal packing forces. Several EM studies of negatively stained myosin II and HMM have confirmed the closed conformation in individual molecules ^{14; 15; 16}. More recently, the same head-head conformation was observed in relaxed phosphorylation-regulated striated muscle thick filaments ^{17; 18; 19}, which led to the conclusion that the interacting-head structure was a fundamental property of the relaxed state of thick filaments in both smooth and striated muscles over a wide range of species ^{14; 16; 20}.

The compact structure for dephosphorylated smM is crucial for down regulating the ATPase activity. Thus, it follows that the active or phosphorylated state characterized by the open, 6S form of myosin ²¹ must differ, but this has not been visualized at the higher resolution offered by electron cryomicroscopy (cryoEM). To determine this structure in greater detail, we formed 2D arrays of expressed thiophosphorylated smHMM on a positively charged lipid monolayer, and used electron crystallography to determine its 3D structure to a resolution of ~25 Å. This structure reveals a matrix of extended dimers of individual smHMM molecules. The heads of individual smHMM monomers have an open or extended

conformation with a head-head placement between two different smHMM molecules bearing some similarity to the intramolecular head-head interaction of dephosphorylated smHMM, but occurring in an *intermolecular* fashion. Despite the overall similarity, there are significant differences from the intramolecular head-head interaction seen in dephosphorylated smHMM. While the intermolecular head-head interaction may be caused by crystal packing forces, it nonetheless suggests an inherent attraction between myosin heads in these areas. Because the heads come from different molecules, it implies that RLC phosphorylation does not alter the characteristics of the binding interface between myosin MDs, but rather alters the myosin head structure in such a way as to affect the ability of the two heads of a single smHMM molecule to adopt the inactive conformation. We propose that the effect of phosphorylation on the inactive conformation is not equivalent for the two heads, and that phosphorylation of the “blocked head” will lead to activation, consistent with the partial activation that occurs when smHMM is phosphorylated on only one RLC ^{22,23}.

Results

The 2-D arrays exceed a μm in size and usually have sufficient contrast when frozen-hydrated to reveal the periodic arrangement of protein molecules in electron micrographs (Supplemental Fig. 1A). Computed transforms of electron micrographs have data of acceptable signal to noise ratio to a resolution of $\sim 20 \text{ \AA}$ though data at resolutions exceeding $25 \text{ \AA}$ are generally weak and probably contribute little to the final reconstruction (Supplemental Fig. 1B). The resolution is comparable to that from 2D arrays of dephosphorylated, frozen-hydrated smHMM ¹². However, compared to the 2-D arrays reported earlier ¹¹ the lattice constants and the molecular packing differ.

The in-plane projection showed strong 2-fold symmetry indicating that the 2-sided plane group was p2 rather than the only alternative (p1) that would be consistent with the unit cell dimensions. Merging of the tilt data was carried out in p2 and fitting of the lattice lines was satisfactory (Supplemental Fig. 2). The 2-sided plane group indicates that the unit cell contains two smHMM molecules. The map is intricate and differs considerably from that of dephosphorylated smHMM.

Atomic Model Flexible Fitting

We used Normal Mode Flexible Fitting (NMFF)²⁴ to conform initial models that consisted of fully connected smHMM atomic models into the density map. NMFF conforms an initial model to a density map by bending the molecule along natural directions of movement as defined by their normal modes, without resorting to any manual bond rotations or displacements over single hinge points. We used four different open conformation models and one closed conformation model for smHMM (shown schematically in Figs. 2A,B). Both models result in similar head-head interactions, in one case *intramolecular* and in the other case *intermolecular* in character. The open conformation models were constructed by combining two homology models for the smooth muscle RLC with either two heptads, 14 residues (2hep), or three heptads, 21 residues (3hep), of non-coiled α -helix at the S1-S2 junction preceding the beginning of the coiled-coil onto a smooth muscle MD-ELC (MDE) structure ²⁵. One set of models was based on the skeletal muscle myosin RLC^{ske} ²⁶ (MDER^{ske}-2hep and MDER^{ske}-3hep), and the other was based on the scallop myosin RLC^{sca} ²⁷ (MDER^{sca}-2hep, and MDER^{sca}-3hep). We also explored a closed conformation model ¹³ but this was fit only using the RLC^{sca} homology model. For the fits using the open conformation here was little difference in the quality with respect to the number of non-coiled heptads preceding the coiled-coil.

Both the MDER^{sca}-2hep and MDER^{sca}-3hep models fit the electron density map well achieving cross correlation coefficients of 0.85 (Fig. 2). This was also the best fit in terms of

the distortion of the initial structure, particularly with respect to the connections of the two heads with the S2 domain and the fit of the S2 domain to the density feature to which it most likely belonged (Fig. 2C). The S2 connection extends smoothly from the hook (a sharp bend in the heavy chain helix where the N-terminus of the RLC binds, see Fig. 1B) into the coiled-coil density with little distortion.

Both MDER^{ske}-2hep and MDER^{ske}-3hep failed to fit, stopped prematurely, and achieved low cross-correlations of 0.34 and 0.41 respectively. The fitting appeared to fail because the direction of the hook of the RLC^{ske} homology model and its connection with S2 takes a trajectory that is too different from the density to be fit by NMFF, probably because this model either lacks low frequency normal modes in the needed directions or the initial open conformation has poor overlap with the experimental density map. The MDER^{ske} could be fit manually by distorting the connection between the hook and the S2 region.

The fit of the closed conformation was done in 2 ways: by fitting the paired heads alone and by fitting the paired heads with a connecting S2 segment. The paired head structures were taken from Liu et al.¹³. Domain movements during the fitting were in the direction of separating the paired RLCs. When this was done without a connecting S2 domain, the RLCs became distorted. When done with a connecting S2 domain, the RLCs distorted less and the correlation coefficient improved slightly. Using either approach, the fit of the closed conformation was a poor match to the density (Fig. 3A). The correlation coefficient dropped to 0.76. The myosin heads fit individually, but left significant volumes of high density unoccupied. Moreover, the crystal contacts were few which would be inconsistent with the formation of large, well ordered arrays. In the fit of the open conformation no large regions of high density were completely unoccupied and contacts were more plentiful between molecules (Fig. 3B).

All-Atom Atomic Model

Building an all-atom model into the fit of the C α representation required assumptions of flexibility. Myosin heads in both skeletal and smooth muscle myosin have demonstrated flexibility in the area between the MD and the LCD^{28; 29}, so HC segment 785-801 was treated as flexible, while the HC segment from 853-900 allowed for flexibility between the S2 region and the LCD. Comparison of the fitted structure with the original revealed some movement where the converter domain joins the MD, and led to the selection of 716-724 as a flexible segment. Despite evidence for rigidity between converter domain and ELC³⁰, the fitting indicated a flexible segment in that location as well. The final model has segments 2-715, 725-784, 802-814, and 824-832 from the starting MDER^{sca} model, with the residues within the connecting flexible segments determined from NMFF rebuilt as all atom structures.

NMFF works best when domains move toward each other during the fitting process, that is during an open to closed conformational change^{31; 32}. The change in the position of the ELC vis-à-vis the converter and MD involves separation of these domains from the highly compact initial model. Consequently, the structure was comparatively more distorted here than has been observed for the flexible fitting of blocked heads in dephosphorylated smHMM.

Preserved Head-Head Interactions

The fit starting from the MDER^{sca}-2hep model reveals an open or extended smHMM molecule, but with head-head interactions occurring between separate smHMM molecules (Fig. 2B,C). The unit cell, which has a two-fold axis perpendicular to the plane of the array, requires a dimeric structure, while the array as a whole consists of a repeating pattern of

these dimers arranged in staggered stripes across its face (Fig. 2B). The pair of smHMMs in a unit cell have an *intermolecular* contact between each of the heads of the two molecules (Fig. 2C). In the dephosphorylated smHMM structure¹² the heads are referred to as “blocked” and “free” depending on their ability to bind actin without steric interference by the companion head. Here we label the heads of phosphorylated smHMM as “blocked-like” and “free-like” to relate them to similar relative positions in the dephosphorylated structure.

The interaction between the blocked-like head of one phosphorylated smHMM with the free-like head of the neighboring molecule resembles that of the closed conformation (Fig. 4A, C). In our phosphorylated model two loops involving residues 371-373 and 384-390 of the blocked-like head contact a loop consisting of residues 742-750 of the converter of the free-like head. The interactions appear to be surface contacts on the outside of each head with only a small amount of buried surface. In contrast, the blocked head of the dephosphorylated atomic model^{12; 13} buries a larger surface by involving the ELC, converter, and 50 kDa domains of the free head (Fig. 4B, D).

For the phosphorylated smHMM, contact with the monolayer occurs primarily through the actin-binding surface of the free-like head, portions of the ELC and RLC of both blocked-like and free-like heads (Fig. 5A). Most of the smHMM mass, including the blocked-like head MD and S2, is held away from the monolayer. The primary interaction with the positively charged lipid monolayer, is due to opposite net charge³³. The smHMM molecule touches the monolayer at HC residues 395-400 (collective pI = 6.0) of the free-like head, residues 3-7 (collective pI = 6.1) on the ELC of the blocked-like head, and residues 76-79 (collective pI = 4.8) on the RLC of the blocked-like head. The position of the free head of dephosphorylated smHMM (Fig. 5B) is more vertical with respect to the monolayer face than that of the free-like head of phosphorylated smHMM (Fig. 5A). Thus, in both the dephosphorylated and phosphorylated arrays of smHMM, the same region of the free or free-like heads and the blocked or blocked-like heads contact the lipid monolayer while the MDs of the blocked or blocked-like head are held off of the monolayer.

A major difference is the S2 position relative to the positively charged monolayer. In the arrays of dephosphorylated smHMM density that could be assigned to S2 when contoured at a low threshold, other than the short segment of strong density adjacent to the neighboring free head, lies between the blocked head and the monolayer¹². This is also the path predicted by previous modeling studies³⁴ as well as by the recent thick filament structures^{17; 19}. In the 2-D arrays of 10S myosin a strong density assignable to the S2 again lays between the blocked head MD and the monolayer¹³. The greater visibility of S2 in the 10S smM arrays probably arises because of stabilization by an adjacent segment of light meromyosin. The poor visibility of the S2 in the smHMM arrays may arise because of degenerate pathways to the neighboring or next nearest neighbor free head (for example, see Figure 2 of³⁴). The short segment of S2 visible in the map in Figure 2 is positioned on the side opposite the monolayer. The rest of the S2 is disordered. The only stabilizing influence seems to be the S1-S2 junction. It has been hypothesized that the free head RLC N-terminus (residues 1-24) might stabilize the S2 within the arrays of dephosphorylated smHMM; different segments of the rod in the 10S smM arrays may be stabilized by the free head RLC N-terminus in the same way³⁴. After phosphorylation, it seems likely that the N-terminus is involved in other interactions.

LCD Atomic Model Comparisons

We compared the LCDs of the present phosphorylated smHMM model, two published dephosphorylated smHMM models^{12; 13}, the tarantula thick filament structure¹⁷, the scallop LCD structures $\pm\text{Ca}^{2+}$ ^{35; 36}, and the MDER^{ske} model, and the MDER^{sca} models from the present work (Fig. 6). Least squares fits were used to align the models at residues

823-835 of the RLC binding site or the equivalent site in the scallop LCD. Several conclusions can be drawn. First, all of the HCs in the LCD models of blocked heads show a clear preference to bend in the same direction and to similar extents at a point of flexibility in the HC between the ELC and the RLC (Fig. 6A). Second, the direction and bend of the HC of the free heads is significantly more variable in direction and extent but the trend is in the opposite direction from that of the blocked heads and the two starting models used for the fitting (Fig. 6B). The two starting models, MDER^{ske} and MDER^{sca} are both similar in this region and fall between the positions of blocked heads and the positions of free and phosphorylated heads. The Ca²⁺-free and Ca²⁺-bound scallop LCDs are very similar and the difference is too small to be conclusive. Both fall between the dephosphorylated blocked head LCD and both of the phosphorylated LCDs. What little difference there is between +Ca²⁺ and -Ca²⁺ is in the same direction as $\pm$ phosphorylation for most of the other models. The Wendt et al. model is the only one fit entirely by hand and the only one that does not follow the trend of the quantitatively fit models for the free heads. Although the correlation is not perfect, generally, the free heads and phosphorylated heads fall on one side of the two starting models (as well as the scallop LCD crystal structures) while the blocked heads fall on the other side.

Discussion

The data presented here constitute the first 3D image of the active, phosphorylated state of smHMM obtained by cryoEM. The atomic model was made by fitting several completely connected smHMM initial models that differed in the reference structure used for the RLC homology model, which in turn was necessitated because the smooth muscle RLC structure is unknown. This was combined with a pair of S2 models that differed in the number of non-coiled heptads at the S1-S2 junction. By using a flexible fitting approach based on normal mode analysis³⁴, we were able to accomplish the fit while keeping the HC of the myosin heads and S2 as a single connected chain. We used the same solution conditions that formed 2D arrays of dephosphorylated smHMM¹², and similar techniques were used to produce the density map. The arrays, although produced under the same conditions, have very different molecular arrangements that could not be fit using the closed, inhibited conformation of dephosphorylated smHMM. An acceptable fit required placing an open head-head arrangement in the lattice in which the pair of HCs from the individual smHMM molecules form a significant interaction with each other only within their S2 dimerization domain and not between their MDs. Nevertheless, the *intermolecular* interaction between a pair of smHMM molecules within the unit cell has a striking resemblance to the *intramolecular* arrangement found in dephosphorylated smHMM.

The intermolecular contacts found in the 2-D arrays of phosphorylated smHMM suggests that the interactions between free-like and blocked-like heads are relatively unperturbed by RLC phosphorylation, which occurs at a location >100 Å away from the MD-MD interaction. This observation also implies that these interfaces are inherently attractive to each other. In the *intramolecular* folding to the inhibited, dephosphorylated state, the pair of smHMM heads (2 heads = 0.33×10^{-23} moles) are confined by virtue of their common S2 domain to a volume of $4\pi (190\text{\AA})^3/3$, (2.87×10^{-20} liters), for a head concentration of 0.13 mM; the interaction to form the closed, inhibited structure is independent of solution smHMM concentration. The pair of phosphorylated smHMM molecules in the arrays (4 heads) occupy a volume of 3.8×10^{-21} liters for a head concentration of 1.7 mM, more than 15x higher. Because the kinetics of intramolecular folding are unimolecular, while the kinetics of intermolecular interaction are bimolecular, a direct comparison is not possible. However, the concentration within the arrays, combined with the possibility that much of the diffusion may be confined to a planar surface, seems to satisfy the concentration

requirement, and at least partially satisfy the steric requirement, for head-head interaction to occur.

The several published structures of dephosphorylated myosin heads were all fit to the density maps by different methods. The first structure¹² was fit entirely manually. A subsequent structure of both smHMM and the 10S smM structure were fit using a crystallographic fitting program, but required assumptions as to the location of flexible hinges¹³. The head structure from relaxed tarantula thick filaments was obtained using flexible fitting, but only for the heads; the S2 was fit separately³⁷. All of these models used either an RLC from the chicken skeletal S1 crystal structure¹², or a homology model derived from the same. This seemed reasonable at the time, because replacing the smM RLC with the chicken skeletal RLC renders the molecule permanently in the inhibited state regardless of RLC phosphorylation³⁸.

The present study used a completely connected myosin HC and two different RLC homology models combined with two different lengths of non-coiled α -helix at the S1-S2 junction. Better fittings were obtained using a homology model based on the scallop myosin RLC than the skeletal myosin RLC. Comparison of the phosphorylated head structures with the published dephosphorylated head structures is thus complicated because of differences in the two RLC homology models and whether a fully connected HMM structure or whether S1 and S2 were fit separately. The LCD of the MDER^{sca} starting model, which provided the best fit to our electron density map, differs from that of MDER^{ske} in one significant way. Within the segment of the HC to which the RLC N-terminal domain is bound, a sharp bend occurs, dubbed the “hook” (Fig. 1B). After the bend, the HC continues into the S2 domain. The azimuthal orientation of the hook relative to the HC segment bound by the RLC C-terminal domain differs by $\sim 27^\circ$ between MDER^{ske} and MDER^{sca} which suggests that the homology model based on the scallop RLC is closer to the smooth muscle RLC structure than is the one based on the skeletal RLC.

Previous fittings to dephosphorylated smHMM maps^{12; 13}, as well as the present atomic model fitting, observed a rotation of the LCD about a hinge between the converter domain and the ELC consistent with previously observed flexibility at this position^{28; 29}. The rotation was minimal for the blocked heads, but was both larger and more variable for the free heads. The flexible fitting of phosphorylated heads shown here also altered the initial models at this location. This region comes directly from the MDE crystal structure²⁵ and was unmodified in all of our initial models, so this departure from the crystal structure may be important.

It is significant that for the blocked head atomic models, the bends in the HC at the ELC/RLC juncture are consistently similar in both magnitude and direction (Fig. 6). The myosin head structures of all the starting models for fitting are also very close to the blocked head structures. In contrast, the free heads of the dephosphorylated head structures and both phosphorylated heads from the present work are both very different and at the same time quite variable.

Scallop adductor myosin shares many similarities with smM in its regulatory properties^{39; 40}. Scallop myosin uses increasing calcium concentration as the activation switch instead of RLC phosphorylation. When crystal structures of the scallop LCD in low and high calcium are compared with the phosphorylated and dephosphorylated LCDs from smooth muscle, the low calcium structure is closer to that of the dephosphorylated LCDs, while the high calcium structure is closer to that of the phosphorylated LCDs although the differences are small in magnitude. Thus, the structural changes in the LCD brought on by phosphorylation follow the same trend observed for scallop myosin, although the magnitude

and variability of the changes are larger. This is possibly due to effects of the head-head interactions that enforce a structure on the LCD within HMM that do not occur with the isolated LCD's.

The dephosphorylated (closed) conformation of smHMM must be comparatively stable to account for the 1000-fold change in ATPase activity on phosphorylation⁴. Full length myosin has an even slower ATPase rate in the inhibited conformation⁴¹ due to stabilization by an additional interaction between the RLC and the myosin rod^{42,14}. Several studies with phosphorylated smM show that phosphorylation increases the distribution of distances between the two heads⁴³, and increases the mobility of the active site and the RLC⁴⁴. Phosphorylation of the RLC causes a structural transition in dephosphorylated smM from the closed 10S conformation to the open 6S conformation, which is compatible with filament formation^{21; 45}. In the filamentous form, smM is active and the heads have the necessary mobility with respect to the S1-S2 junction to bind to actin. Flexibility implies that the pair of heads can adopt a wide range of relative positions, which is generally observed for myosin heads under conditions where the closed conformation does not form⁴⁶. The arrangement of heads with respect to S2 in phosphorylated smHMM is thus not a unique conformation, but rather one of many states that phosphorylated smHMM can adopt.

Can structural changes within the individual heads of phosphorylated smHMM suggest a mechanism for disrupting the interactions in dephosphorylated smHMM? We examined this question in two ways. First, we aligned by least squares fitting the free-like and blocked-like heads of *phosphorylated* smHMM to segments of the LCD of *dephosphorylated* smHMM atomic models¹³. After alignment to the LCD, the MD of the dephosphorylated blocked head is oriented significantly downward from its position within the pair of phosphorylated heads and closer in position to the free-like head MD than the blocked-like head MD (Fig. 7A). Conversely, after alignment, the MD of the dephosphorylated free head is positioned not nearly as much downward as was the blocked head MD and again closer in position to the free-like head MD than the blocked-like head MD (Fig. 7B). Thus, phosphorylated smHMM heads most closely resemble free heads of the closed inhibited conformation, which in turn suggests that phosphorylation has variable effects on the free and blocked heads.

We suggest that phosphorylated free heads may be compatible with the closed inhibited conformation, whereas phosphorylated blocked heads are not. In smHMM, the folding pathway is degenerate in that free and blocked heads of the folded conformation are not predisposed. If, in the pathway to folding, the phosphorylated head was destined to become the free head, folding to the inhibited conformation would succeed and the molecule would be inactive for some period of time, which may be shorter than the stability of completely dephosphorylated smHMM. Conversely, if the phosphorylated head was destined to become the blocked head, folding would be unstable. Asymmetry in the effect of phosphorylation may explain why smHMM phosphorylated on only one of its two heads appears to exist in an equilibrium between a fully active state and an inhibited conformation^{22; 23; 47}.

When observed in thick filaments, the closed conformation always has the same orientation^{17; 18; 19}. This suggests that, in thick filaments, the role of free head and blocked head are not interchangeable as they are in HMM, but are instead predetermined. Phosphorylation of the predetermined blocked head would be sufficient to disrupt *intramolecular* head-head interactions and activate the entire molecule whereas phosphorylation of the predetermined free head would have less effect. Thus, phosphorylation of blocked heads would provide a larger population of activated molecules at low levels of phosphorylation than if both heads required phosphorylation. Such a

phenomenon would allow more rapid activation of the smooth muscle cell upon stimulation because only blocked heads need be phosphorylated to achieve activation.

Materials and Methods

Protein preparation

Chicken smHMM was expressed, isolated, and thiophosphorylated as described previously^{11; 40}. Crystallization was carried out at 4°C on a positively charged lipid monolayer system. Crystallization buffers consisted of 1 mM Mg²⁺, 20 mM phosphate, pH 7.8, 1 mM ATP, 1 mM EGTA, 7-10% polyethylene glycol 6000, and 90-120 mM NaCl¹¹.

Electron Microscopy

Specimens were plunge-frozen for electron cryomicroscopy as described⁴⁸ and examined on a FEI (Eindhoven, The Netherlands) CM300-FEG electron microscope at 300 kV accelerating voltage using low-dose procedures. Micrographs were recorded on Kodak SO-163 film and developed for maximum electron speed. Micrographs were screened initially by optical diffraction and digitized on a Zeiss Photoscan 2000 scanner at a resolution of 4.6 Å/pixel with respect to the original object. ICE image processing software⁴⁹ was used to process the micrographs with crystal unbending⁵⁰ and subsequent merging accomplished as previously described¹².

The structure factors were measured from 85 images tilted to as high as 60°. The average unit cell dimensions derived from images of untilted specimens are $a = 219.3 \pm 4.0$ Å, $b = 174.8 \pm 5.0$ Å, and $\gamma = 94.4 \pm 0.7^\circ$. The thickness is 100 Å. These lattice constants differ from those reported earlier¹¹. Computed transforms of electron micrographs usually show visible spots to ~30 Å (Supplemental Fig. 1B) and have measurable spots of $IQ \leq 4$ (a measure of signal-to-noise ratio in electron crystallography) to a resolution of ~20 Å though data at >25 Å are generally weak compared with those on the inner lattice lines. Structure factors were refined to a common phase origin and merged in the two-sided plane group p2 with an average phase residual of 17.9°.

Model Building

Putative active-state models of smHMM were built from known crystal structures and experimental constraints using homology modeling as previously described³⁴. The ATP-bound smooth muscle S1 structure was built from the MDE structure PDB-1BR1²⁵, with two different homology models for the RLC and its bound segment of HC. One S1 model, MDER^{sca}, was based on the scallop myosin LCD, PDB-1WDC²⁷, and the other, MDER^{ske} on the chicken skeletal muscle S1, PDB-2MYS²⁶. The model based on the PDB-2MYS was built using RLC coordinates with side-chains built in, kindly provided by Dr. Ivan Rayment. The major difference between the MDER^{ske} and MDER^{sca} homology models is in the bend in the HC between the ELC and RLC and in the RLC structure itself, which changes the angle of the HC residues 840-851, dubbed the “hook”, by ~27°. The RLC homology models, were first placed on their parent LCD structure and then aligned onto the smooth muscle MDE structure using heavy chain residues 793-814. All of the residues from the MDE structure (except for the flag tag) were retained when the two structures were merged. Homology modeling and all other model manipulations were done using the MMTSB tool set⁵¹.

The α -helical coiled-coil structure of the S2 domain was modeled after its predicted structure using a program supplied by Dr Gerald Offer⁵². Two models of the S2 domain were constructed with either two or three heptads of non-coiled α -helix at the S1-S2 junction. By combining the two homology modeled active state structures with the two

different S2 domains, four active state models were produced, each based on the MDE structure from PDB - 1BR1, as follows: two with the 2MYS RLC homology model and either 2 or 3 heptads of non-coiled α -helix at the S1-S2 junction, MDER^{ske}-2hep, MDER^{ske}-3hep, and likewise with the 1WDC RLC homology model, MDER^{sca}-2hep, MDER^{sca}-3hep.

A rough fit of the model to the electron density map was accomplished using the X-ray crystallography modeling program O⁵³, and the density map masked using the program MAMA⁵⁴. Since the initial fitting of atomic model to the electron density map involved large conformational changes from the symmetric starting model; the Ca backbone of the initial model was iteratively fit to the density using NMFF^{34; 55}. The resulting Ca structure was then compared with the original atomic model in a segmented fashion using the MMTSB toolset.

An all heavy atom structure was then fabricated as follows: (1) where the original structure and NMFF structure had a RMS deviation $<0.5\text{\AA}$, the original structure from either the crystal structure or the RLC homology model was used. (2) Segments having a larger RMS deviation were converted into an all-atom model of that segment using complete.pl from the MMTSB toolset. (3) The entire structure was then minimized using minCHARMM.pl from the MMTSB toolset⁵¹ with the segments from the original structure constrained. We did not model the 24 residue, N-terminus of the smooth muscle RLC, the corresponding segment of which is not visible in either PDB-2MYS or PDB-1WDC.

Accession Numbers

The structure factors have been deposited in the EBI data base under accession number EMD-5257. The atomic coordinates have been deposited in the PDB under accession number 3J04.

Supplementary Material

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Glossary of Terms

smHMM	smooth muscle heavy meromyosin
smM	smooth muscle myosin
ELC	essential light chain
RLC	regulatory light chain
MD	motor domain
S2	α -helical, coiled-coil dimerization domain of myosin
S1	also called the myosin head consisting of motor domain and lever arm
NMFF	normal model flexible fitting

2hep/3hep number of heptads of non-coiled α -helix (7 residues) at the beginning of the S2 domain

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Figure 1. Background on smooth muscle myosin regulation

(A) The closed conformation of smM that inhibits actin activated ATPase activity. The HC of the blocked head is colored red and for the free head is colored magenta. The ELC is blue and the RLC is orange. This is the same coloring scheme used throughout. The asymmetric arrangement places the blocked head MD onto the free head MD in a position where it contacts both the free head converter domain, HC and ELC. The placement results in the free head sterically inhibiting actin binding by the blocked head, but actin binding by the free head is not similarly affected. The myosin head-S2 junction and the S2 domain would be on the near side of the molecule in this view. (B) The LCD of the the starting model. The ELC and its bound segment of HC comes from the MDE crystal structure; the RLC and its segment of HC is a homology model based on the scallop LCD. The first residue of the RLC N-terminal domain is F25, which is shown as a CPK model. Within the RLC N-terminal domain, the HC makes a nearly 90° bend to form the “hook” which connects the myosin head to the coiled-coil S2 domain. The hook points up in Panel A. Panel A produced using UCSF-Chimera ⁵⁶, panel B produced using VMD ⁵⁷.

Figure 2. Electron density map of phosphorylated smHMM

The color scheme for all atomic models, unless otherwise noted, is as follows: the HC of the free-like myosin head is magenta, and the HC of the blocked-like head is red. For both myosin heads, the converter domain is green, the ELC blue, and the RLC orange. (A) Averaged in-plane projection of frozen hydrated arrays with a surface view of the 3D reconstruction as *Inset*. Outlined are two possible fits of the phosphorylated smHMM model: I (magenta) *intermolecular* head-to-head contact; II (green) *intramolecular* head-to-head contact. The view orientation is perpendicular to the plane of the crystal. The lipid monolayer would be located below the structure in this view. (B) View of the open smHMM atomic model in space filling, and dimers of the open intermolecular head-to-head contact model (shown in ribbon view in light blue and magenta). (C) A pair of models from the best fit of the open configuration from MDER^{sca}-2hep smHMM. Same coloring scheme used here as for the space filling model shown in (B). Contour threshold set at 140 (arbitrary units).

Figure 3. Comparison of the fitting of the closed conformation model with the open conformation model

Adjacent molecules of the same model are also shown. (A) The fit using NMFF of the dephosphorylated closed conformation¹³. A single smHMM molecule is outlined. Two regions of density that are not fit by any feature of the model are marked with asterisks (*). There is no density in the map that can be assigned to an S2 domain for this model. (B) Similar display of the best open conformation model with adjacent molecules shown and one molecule outlined. This interpretation assigns all significant densities to at least one model feature and has a density that can be assigned to S2. This interpretation also has better crystal contacts.

Figure 4. Comparison of the head-to-head interactions of both phosphorylated and dephosphorylated smHMM

(A) View perpendicular to the crystal plane showing the intermolecular interaction between phosphorylated smHMM molecules. The free-like head sequences contributing to the interaction include residues 742-750 (at the end of a disordered loop) and blocked-like head residues contributing include 371-373 and 384-390. (B) The interaction of the dephosphorylated smHMM molecule in a view similar to (A) shows intramolecular binding, where the blocked head binds intimately into the area between the converter and ELC of the free head¹³. (C) View parallel to the crystal plane showing the intermolecular interaction of phosphorylated smHMM. (D) The interaction of the dephosphorylated smHMM molecule in a view similar to (C) shows intramolecular binding, where the blocked head MD binds intimately into the area between the converter and ELC of the free head¹³. The potential areas of interaction for the phosphorylated heads are shown as spheres centered on the C α atom for clarity.

Figure 5. Phosphorylated and dephosphorylated smHMM oriented as if resting vertically on the lipid monolayer

All views show molecular models fitted to reconstructions (gray) viewed within the crystal plane. The rows of “clothes pins” represent the position of the lipid monolayer. (A) The model of two phosphorylated smHMM molecules from one unit cell shown with their head contacting *intermolecularly* with the lipid monolayer shown schematically. Direction of view is almost left to right in Figure 2 but along the plane of the array. (B) View of the Wendt et al. model of dephosphorylated smHMM, with the heads bound *intramolecularly*. The interaction surface in this case is larger than for phosphorylated smHMM. Depth of displayed map is $\sim 85\text{\AA}$.

Figure 6. Comparison of various smooth and scallop muscle LCD models

(A) Side views and end views after rotation by 90° of the HC component of LCD models from the blocked heads of various smooth muscle reconstructions aligned at residues 823-835, or to the corresponding residues in the case of the scallop LCD. (B) Corresponding views for the free heads. The scallop LCD $\pm\text{Ca}^{2+}$ is also shown for comparison. The color scheme for HC diagrams is as follows: the blocked heads from the dephosphorylated smHMM models of ^{12; 13} and tarantula ³⁷ are red, the HCs of the corresponding free heads are magenta; the free-like head (this work) is blue, the blocked-like head is yellow; in (A) the HC of the initial MDER^{sca} homology model is cyan and the HC of the MDER^{ske} initial model is light green; in (B) the HC of the scallop LCD in low $[\text{Ca}^{2+}]$ is gray and in high $[\text{Ca}^{2+}]$ is black.

Figure 7. Comparison of phosphorylated and dephosphorylated HCs

All HCs are aligned to residues 780-835 (LCD) of their blocked and blocked-like heads (A) or free and free-like heads (B). Coloring scheme has the free head of the closed, dephosphorylated smHMM - magenta, blocked head - red, free-like head of phosphorylated smHMM - blue, blocked-like head - yellow. Dephosphorylated smHMM atomic model from Liu et al. while the phosphorylated heads are from the MDER^{sca}-2hep model. The diagram shows that the free head of dephosphorylated smHMM is much closer in structure to the free-like head of phosphorylated smHMM than is the blocked head.