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An unstable head-rod junction may promote folding into the compact off-state conformation of regulated myosins

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SUMMARY

The N-terminal region of myosin's rod-like subfragment 2 (S2) joins the two heads of this dimeric molecule and is key to its function. Previously, a crystal structure of this predominantly coiled-coil region was determined for a short fragment (51 residues, plus leucine zipper) of the scallop striated muscle myosin isoform. Here, the N-terminal 10-14 residues were found to be disordered. We have now determined the structure of the same scallop peptide in three additional crystal environments. In each of two of these structures, improved order has now allowed visualization of the entire N-terminus in one chain of the dimeric peptide. We have also compared the melting temperatures of this scallop S2 peptide and analogous peptides from three other isoforms. Taken together, these studies, along with examination of sequences, point to a diminished stability of the N-terminal region of S2 in regulated myosins, compared to those myosins whose regulation is thin filament-linked. It seems plain that this isoform-specific instability promotes the off-state conformation of the heads in regulated myosins. We also discuss how myosin isoforms with varied thermal stabilities share the basic capacity to transmit force efficiently in order to produce contraction in their on states.

Keywords

Alpha-helical coiled coil; Myosin Subfragment 2; Off State; X-ray diffraction; Thermal Stability

INTRODUCTION

Conventional myosin (myosin II) is the motor that drives muscle contraction¹, and whose mechanism exemplifies the transduction pathway of an allosteric protein². The molecule consists of a long two-chain alpha-helical coiled-coil rod whose C-terminal region assembles to form the thick filament backbone, and whose N-terminal subfragment-2 (S2) rod region swings out to allow the two attached subfragment-1 (S1) heads (Fig. 1a, left) to interact with

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F-actin of the thin filament. Each S1 head comprises a motor domain (MD), which has the binding sites for F-actin and a nucleotide, together with a light-chain-bearing “lever arm” (also called the regulatory domain (RD)) (see 3). Comparatively small movements among three of the relatively rigid subdomains of the MD (induced by the binding of nucleotide and/or actin) are amplified by its fourth subdomain (the “converter”) and the adjoined lever arm by means of short flexible linkers between them⁴. The resulting force is transmitted through S2 to produce the sliding motion between the thick and thin filaments (see 5 for recent review). Contraction and other myosin-dependent functions are turned “on” and “off” by changes in Ca^{2+} concentration, which lead to changes in the conformations of certain regulatory molecules. This switch involves either so-called “non-regulated” myosins, where the regulatory molecules are on the thin filament (as in vertebrate skeletal and cardiac muscles) (see 6 for recent review) and/or “regulated myosins” which themselves change conformation to control contraction (as in vertebrate smooth-muscle and non-muscle myosin II⁷ as well as that in molluscan striated and smooth muscles⁸) (see also⁹).

The nature of the junction between myosin’s two S1 heads and its S2 rod appears to be critical both for the efficient transmission of force production during the contractile cycle¹⁰ as well as for the conformational changes in vertebrate smooth and scallop striated muscles necessary for their regulation^{11; 12; 13}. It is the apparent capacity of this junction to adopt different structures, as well as the intrinsic marginal stability of these conformations, that appear to be essential for function. Although relatively high-resolution and complete crystal structures of scallop S1 and the lever arm (2; 14; 15; 16; 17; 18) on one side of this junction and of a short N-terminal peptide construct of scallop S2¹⁹ on the other side have been determined, an atomic description of the intact head-rod junction in any of its states has yet to be determined. Moreover, the N-terminal 10-14 residues of the scallop S2 peptide construct, i.e. those residues of S2 closest to the location of the head-rod junction, were found to be disordered¹⁹, but have recently been visualized in a long S2 peptide from a non-regulated human cardiac myosin²⁰.

Here we report new crystal forms of the scallop S2 peptide construct, in which this N-terminal region is now relatively ordered, as well as thermal stability experiments of this S2 peptide and analogous constructs from three other myosin isoforms. These studies, together with sequence comparisons, point to a diminished stability of the N-terminal region of S2 in regulated myosins compared to those myosins whose regulation is thin filament linked. These results help provide a physical basis for the formation of the compact off-state conformation observed for regulated myosins. Other key structural aspects of S2 are also discussed, including an improved definition of the head-tail junction, the significance of two-fold symmetry in S2 structures, and consideration of structural stabilization of this junction in order to transmit tension.

RESULTS

Overall Structure of Scallop S2N51

We have now determined the structure of the 51 pairs of residues of scallop myosin’s rod (S2) adjacent to the head-rod (S1-S2) junction in a total of five different crystal packing environments (Fig. 1A). In addition to residues 835-885 of the myosin heavy chain from the bay scallop *Argopecten irradians*, the chimeric peptide that was used for crystallization also contains a tetrapeptide tag at the N-terminus, and a Gly-Ser linker and “leucine zipper” at its C-terminus (Fig. 1B) (see Methods for sequence and further details). Previously, this peptide (called “S2N51”) was crystallized at 16°C, and, after flash-freezing, its structure was determined to 2.5-Å resolution in the orthorhombic space group $P2_12_12_1$ with two molecular dimers (“AB” and “CD”, hence 4 “independent” chains) in the asymmetric unit. In that structure, residues 835-842 (and the tetrapeptide tag) at the N-terminus were found to be disordered, and residues 843-846 displayed very high B factors. We have now crystallized this

peptide using various solutions at 4°C, and (after flash freezing) have determined its structure in the same orthorhombic space group as previously but at lower (3.0 Å) resolution, and to a higher 2.3-Å resolution in each of two new space groups: centered monoclinic C2 (Fig. 1C) and primitive monoclinic P2₁ (see Figs. 2 and 3) with one and two molecular dimers per asymmetric unit respectively (additional 6 independent chains). (See Methods and Table 1 for crystallization conditions and data statistics.)

The new information, provided by one of the P2₁ chains and one of the C2 chains, is that all of the residues at the N-terminus of a scallop S2 chain can now be seen (Fig. 2). Their improved order appears to be a result of crystal packing effects rather than crystallization temperature since this region is disordered in the P2₁2₁2₁ space group whether crystallized at 4°C or 16°C. The two well ordered chains in the newly obtained space groups adopt an alpha-helical conformation (with low B-factors) from residue 885 back to residue 838. In contrast, residues 835-837, corresponding to the proline, leucine, leucine that form the head-rod junction in native myosin, adopt an extended conformation in the current scallop S2 peptide. This segment is oriented away from the coiled-coil axis that follows in one of the two packing environments, but back towards the axis in the other. Despite the presence of the alpha helix-inhibiting proline 21; 22, these three residues adopt a near alpha-helical conformation (somewhat bent at the proline) in some of the scallop S1¹⁷ and scallop¹⁴ and *Physarum polycephalum*²³ lever arm (RD) structures; here they are apparently stabilized in this conformation by a hydrophobic knob-into-hole-type interaction of leucine 837 with helix A of the regulatory light chain (RLC) (Fig. 3a). The heavy chain may thus be alpha helical through the head-tail junction in certain states of native (i.e. RLC-containing) scallop myosin, and our current S2 structures suggest possible conformations of these residues when the RLC is absent. (Note that an extended conformation is seen in scallop S2 for residues 835-837 whether the tetrapeptide tag is ordered (C2 chain) or not (P2₁ chain).) Note also that residues 835-837 as well as the residues N-terminal to 835 are quite conserved among muscle myosin heavy chains, whereas the region of the heavy chain beginning with residue 838 is quite variable (see fig. 3c). This pattern in the sequences along with the current scallop structures suggest that it is the connection between leucine 837 and residue 838, rather than proline 835, that more accurately defines the boundary between the head and rod of native myosin. [Knowledge of this boundary could be important, for example, for mutational experiments that aim to relate S2 coiled-coil stability to functional aspects (e.g. actin binding, mechanical performance) of myosin^{10; 24}; for example, any mutation of S2 that includes changing leucine 837 may unintentionally alter the stability of S1 as well.]

For the rest of the scallop S2N51 coiled coil, the structures in the new P2₁ and C2 space groups generally confirm and extend many of the observations made previously for the molecule in the P2₁2₁2₁ space group¹⁹, especially concerning the flexibility and stability of the structure. Some of the features found among the four chains of the P2₁2₁2₁ space group and indicative of scallop S2's flexibility appear less pronounced among the 6 independent chains of the new P2₁ and C2 space groups. In addition to the improved order at the N-terminus, there is somewhat decreased variability in the conformations of the α -position lysines and in the bending of the coiled coil (see fig. 2 legend). Nevertheless, most of the structural features in the new space groups remain consistent with a relatively flexible and unstable coiled coil. For example, the increase in the diameter of the coiled coil from 10 Å to 11 Å between residues 857 and 849¹⁹ also occurs in the new crystal forms, and here the trend continues to residue 841 where the helical axes are between 11.5 and 12 Å apart. A divergence of the alpha helices near the head-rod junction has been predicted in models of two-headed myosin (see 25). Moreover, in this region, as the coiled-coil diameter increases, so does the local pitch (as measured by TWISTER²⁶) from close to 156 Å at residue 857 to between 195 Å and 223 Å at residue 844. As a result, glutamine 842 in the N-terminal core layer, observed only in the current monoclinic crystal forms, makes tenuous knobs-into-holes interactions, and a coiled coil with canonical knobs-

into-holes interactions begins only at residue 845. Many of the core residues further down the coiled coil are either poorly packed (e.g., α -positions lysines 867 and 881), asymmetrically packed across the coiled coil two-fold axis (e.g. amide and methionine side chains at 846,849,853,856, and 874), and/or are apolar side chains exposed to solvent (including the d -position leucines 870, 877, and 884) due to the lack of interhelical g - e' salt bridges (see 19 and figures therein). Also note that there is relatively little axial stagger between the alpha-helical chains (Fig. 4 and see below).

Comparative thermal stability studies of different isoforms using circular dichroism and inspection of sequences and structure

Previous observations using fragments of different lengths have suggested that scallop S2, including its N-terminal region, is particularly unstable relative to other isoforms^{19; 20; 27}. We have now applied circular dichroism spectroscopy to chimeric fragments of equal length corresponding to S2N51 (with the attached leucine zipper) from four different isoforms of myosin, including scallop, to compare thermal stabilities quantitatively (Fig. 3b and see Methods). The results indicate that the observed melting temperatures for these S2N51 chimeras from non-regulated cardiac (69.3°C) and skeletal myosins (66.6°C), are considerably higher than for those of the S2N51 chimeras from regulated vertebrate smooth myosin (62.3°C) and especially scallop myosin (51.3°C). Similar results have also been obtained from preliminary differential scanning calorimetry studies (data not shown). Moreover, these short S2N51 fragments from the regulated myosins do not dimerize without the attached leucine zipper^{27; 28}, whereas weak dimerization ($T_m = 35.6^\circ\text{C}$) is observed for the leucine zipper-free cardiac S2N51 (data not shown).

These comparative thermal stability results for S2N51 (scallop < vertebrate smooth < vertebrate skeletal < human cardiac) are well correlated with certain features in their sequences (fig. 3c) and available three-dimensional structures (fig. 3a). As discussed previously¹⁹, salt links between alpha helices in a coiled coil can often be stabilizing factors, especially those linking the g and e' positions^{29; 30}, and probably also those that involve more than two charged residues, i.e. complex salt links³¹. Examination of sequences shows that the concentration of potential interhelical salt links and their proximity to the head-rod junction are correlated with the above experimentally derived relative stabilities of the different isoforms of S2N51. The N-terminal region of S2 from scallop has no potential for interhelical salt links. Vertebrate smooth may form g - e' and d - e' salt links about two heptads away from the head-rod junction. Vertebrate skeletal muscle may form g - e' salt links within one heptad of the head-rod junction. Human cardiac may form both g - e' and d - e' salt links within one heptad of the head rod junction. This d - e' link is in fact observed as a strong interhelical complex salt link (Fig. 3a) in the recently determined crystal structure of a long N-terminal fragment of S2 from human cardiac (non-regulated) myosin²⁰. It appears that this link is important in promoting the relatively high order observed for the N-terminal region of this isoform: here the structure is ordered back to the invariant proline in 4 of the 6 crystallographically independent chains (rather than only 2 of 10 in the scallop structures), and these chains, even without RLC stabilization, are entirely alpha helical (in contrast to the extended conformations for residue 835-837 in the two scallop S2N51 chains). Note also that among regulated myosins, the vertebrate smooth sequences also show possible d - e' salt links, albeit two heptads from the head and containing lysines instead of arginines (Fig. 3c); it remains to be determined whether they contribute to the increased thermal stability of S2N51 in these isoforms relative to scallop (fig. 3b).

DISCUSSION

Functional comparison of S2's from non-regulated and regulated myosins: the off state

The role of S2 in regulated myosins appears to be different from that in myosins whose regulation is thin filament linked. The low level of stability in the N-terminal region of regulated myosins described above may be an important factor in their capacity to adopt the off-state conformation. This conformation has been observed at low resolution in electron microscopic studies of two-dimensional crystals or individual molecules of heavy meromyosin and myosin from vertebrate smooth muscle^{11; 32; 33} and more recently in the relaxed thick filaments of invertebrate (tarantula) striated muscle³⁴, both of which are regulated by phosphorylation of the RLC. A similar conformation is probable for the off-state of scallop myosin^{12; 13; 35}, which is regulated by direct binding of calcium to the first EF-hand motif of the ELC near the ELC/RLC interface. This conformation of the dimeric molecule in the off state is quite distinctive and is marked by two major and related features: a compact structure, and an asymmetric interaction between the two heads (Fig. 1d).

Electron microscopic studies, although at low resolution, suggest that near the head-rod junction, the heads bend back towards the rod to permit the compact conformation³⁴. (An untwisting of the coiled coil near the head-rod junction has also been suggested from normal mode analysis³⁶.) Our current X-ray crystallographic, sequence, and circular dichroism studies described above all indicate a relatively unstable N-terminal region of S2 in regulated myosins. Moreover, it appears that the span of the heavy chain through the head-rod junction, especially in scallop myosin, may form a (near-)alpha-helical conformation only when bound to the RLC. Note that, as described above, the mechanism of activation in regulated myosins involves the binding of ions (Ca^{2+} in the case of scallop myosin and Pi in the case of vertebrate smooth-muscle myosin) to or near the RLC, and it is possible that structural effects on this light chain by the release of the ions during inactivation may be transmitted to the adjacent head-rod junction. These destabilizing features of S2 in regulated myosin at or near its head-rod junction may facilitate the bending of this region and the formation of the off-state conformation. In contrast, as shown above, the N-terminal region of S2 appears to be relatively stable in non-regulated myosins such as vertebrate skeletal and especially cardiac isoforms, and would probably adopt highly bent conformations fairly infrequently. These myosins are indeed observed in electron microscopic and single particle image processing studies to adopt the compact "bent-back" conformation less frequently than do regulated myosins (R. Craig, personal communication and³⁷). In fact, these skeletal and cardiac myosins have been observed to form the compact head-head interactions in high numbers only in the presence of specialized agents such as blebbistatin (R. Craig, personal communication and³⁷), which binds in the 50 kDa cleft of the MD and blocks ATPase activity^{38; 39}.

The asymmetric interactions between the two heads of regulated myosins sterically blocks the actin binding site on one head and restricts the capacity of the other head to undergo the power stroke. In this compact off-state conformation, the part of S2 about 60-80 residues from the head-rod junction appears to make contact with the head region^{33; 34}. The recent structure of a long S2 from non-regulated human cardiac myosin shows axial staggering between the two alpha helices in the N-terminal 40 residues (Fig. 4) and a resulting bend between this region and the following in-register coiled coil²⁰. It has been suggested that such asymmetric features of S2 may be a factor promoting non-equivalence of the S1 heads²⁰, which occurs in the off state of regulated myosins. The alpha helices in S2N51 from scallop (regulated) myosin, are, however, relatively unstaggered (fig. 4). It is thus not yet clear whether S2 asymmetry plays a role in the off state of regulated myosins.

Additional functional aspects of order and disorder near the head-rod junction: the on state

Weakness at or near the N-terminus of the S2 coiled coil, evident for residues 838-845 in each of the S2N51 peptide structures determined, is an important aspect of myosin's design¹⁹. In addition to its potential role in promoting the compact off state of regulated myosin (discussed above), instability in this region may also promote optimal mechanical performance in the on state (see 10).

Nevertheless, the entire span of any isoform of S2, including the residues immediately adjacent to the head-rod junction, must also be capable of adopting a rigid enough conformation to transmit force efficiently in order to produce contraction in its on state^{20; 40}. (A recent modeling study of smooth-muscle myosin also suggests that the head-rod junction maintains an alpha-helical nature, albeit not in a coiled coil, during the transition from the on to the off state³⁶.) The crystallographic results show an alpha-helical structure immediately adjacent to the head-rod junction (from residue 838) in S2 fragments from regulated (current scallop structures) as well as in a non-regulated (human cardiac²⁰) myosins. In intact native myosin, the alpha-helical conformation of these S2 residues closest to the head-rod junction, i.e. residues 838-841, may be further promoted by the local stabilization of (alpha) helical conformations of the main chain on both sides of this segment: As described above (and see fig. 3a) a (nearly alpha-) helical conformation of the main chain of S1 up to residue 837 is promoted by contacts with the RLC, and the alpha-helical conformation of S2 is stabilized by the coiled coil with strong knob-into-holes interactions between the two heavy chains that start at about residue 842-845. With an entirely alpha-helical model for the heavy chains through the head-rod junction, S1 heads extend out nearly perpendicularly and away from the S2 coiled-coil axis (fig. 1a), as previously modeled (see 25). Such a model is generally consistent with the on state of double-headed myosin where the two heads of myosin can be found in various conformations relative to one another and to the rod, including this "two heads out" model¹². On-state myosin found in other conformations may require some melting of the coiled coil near the head-rod junction.

We have described above a link between the isoform-specific differences for S2 near the head-rod junction and different functional requirements between isoforms to form a compact conformation in the off state. But the sequence differences between isoforms would also be expected to influence the properties of the head-rod junction in the active state as well. Perhaps the low level of stability in scallop myosin, compared to say vertebrate smooth-muscle myosin (fig. 3b), relates to the relatively low temperature in which this organism lives⁴¹. Alternatively, this difference in stability may relate to the different myosin-linked regulatory mechanisms (direct Ca^{2+} binding to the ELC versus phosphorylation of the RLC) that these muscles display.

Coda

Myosin is designed to function in a number of conformational states, and different isoforms (now more than about 25 classes⁴²) have evolved for various specialized kinds of activity. Within the large myosin II class, the actin-binding and enzymatic "head" (S1) of the molecule displays a number of such conformational variations – especially in the lever arm. Here we have shown that the S2 region of the tail of the molecule nearest the head is also specialized in structure. One of the key states of myosin is the "resting" or "off" state. Recent low-resolution structural results for this state have shown a "folded-back" compact conformation of the two heads, which are bound to one another in an asymmetric pattern. Atomic resolution (crystallographic) structures are now being sought in order to define the precise head-to-head interactions that produce this state, and to visualize, as well, the role of the S2 region and the rest of the rod in promoting or reducing the stability of this state.

MATERIALS AND METHODS

Crystallographic Studies

The chimeric peptide that we have crystallized (Fig. 1) consists of: Gly-Ser-His-Met at the N-terminus, followed by residues 835-885 of the myosin heavy chain from the bay scallop *Argopecten irradians* (i.e. the first 51 residues of the S2 domain that we call “S2N51”), a Gly-Ser linker, and residues 250-281 (the “leucine zipper”) of the yeast transcription factor GCN4. The final sequence of “S2N51/GCN4” is GSHM (the N-terminal tag) followed by PLLSIARQEEEMKEQLKQMDKMKEDLAKTERIKKELEEQNVTLLLEQKNDLF (S2N51) followed by GS (linker) and then MKQLEDKVEELLSKNYHLENEVARLKKLVGER (the leucine zipper). (Note that the leucine zipper promotes formation of the S2N51 coiled coil but is not expected to perturb its local structure owing to the linear nature of the coiled coil motif and decoupling of the structural details provided by the gly-ser linker.) The expression of this chimeric peptide in *E. coli*, its sequential purification on nickel-affinity, MonoQ ion-exchange and Superdex 200 (Pharmacia) columns, its crystallization in the orthorhombic space group $P2_12_12_1$ at 16°C, its data collection at 100K, and structure determination to 2.5-Å resolution has been described previously (see Supplemental material in ref. ¹⁹). We have now repeated vapor diffusion crystallizations of this peptide construct at 4°C. These experiments have yielded an orthorhombic $P2_12_12_1$ space group very similar to that published previously albeit yielding lower resolution data, and two new relatively well-diffracting monoclinic crystal forms (see Table 1 for data collection and refinement statistics)

Orthorhombic crystals at 4°C were obtained from many conditions. We used that obtained by adding 2 µl of protein solution ((4 mg/ml) in 30 mM MOPS buffer, pH 7.2, 40 mM NaCl, 2 mM NaN₃) to 2 µl of 25% PEG 3350, 6% MPD, and equilibrating against 15% PEG 3350, 3.6% MPD, 18 mM MOPS, 24 mM NaCl. They were cryoprotected in 25% PEG 3350, 24 mM NaCl, 18 mM MOPS (pH 7.2) and 20% MPD. Data from a single crystal were collected at 100K to 3.0 Å resolution at the Brookhaven National Laboratory synchrotron (beamline X25, $\lambda=0.9792$ Å). Data were reduced with the HKL data processing system ⁴³. The previous scallop S2 structure (19; pdb id code: 1NKN) was positioned into these data using PHASER ⁴⁴ and refined using CNS_SOLVE version 1.1 ⁴⁵. Minor rebuilding was performed using the program O version 8.0 ⁴⁶. The poorer resolution of these orthorhombic data compared to those obtained previously limits the reliability of any detailed comparison of their structures. Nevertheless, it is clear that the overall features of its structure, in particular the complete disorder at the N-terminal region (see text), remain the same for the orthorhombic crystals produced at 4°C and 16°C.

Crystals belonging to the monoclinic space group $P2_1$ were obtained by mixing 2 µl of protein solution (as above) with 2 µl of 25% PEG 3350, 50 mM NH₄I and equilibrating against 1 ml of 17.5% PEG 3350, 35 mM NH₄I, 28 mM NaCl, 2 mM NaN₃, 20 mM MOPS, pH 6.2. Harvested crystals were cryoprotected in 25.5% PEG 3350 and 15% glycerol. Data from a single crystal were collected at 100K to a resolution of 1.95 Å resolution at the Brookhaven National Laboratory synchrotron (beamline x26c, $\lambda=0.9791$ Å). Data were reduced with the HKL data processing system version 1.97.2 ⁴³. There are two molecules in asymmetric unit. The structure was solved by the molecular replacement method using AMoRe ⁴⁷ and the previous scallop S2 structure (pdb id code: 1NKN) was used as a search model. The structure was rebuilt in program O version 8.0 ⁴⁶ and refined to 2.3 Å resolution with CNS_SOLVE version 1.1 ⁴⁵. As described in the text, the order at the N-terminus is somewhat improved over that observed in the orthorhombic crystal structures. This structure has been deposited into the Protein Data Bank (PDB code: 3BAT).

Crystals belonging to the monoclinic space group C2 were obtained by adding 1.5 μ l of protein solution (as above) to 3 μ l of 16.6% PEG 3350, 4.6% MPD, 13 mM NaCl, 2 mM NaN₃, 10 mM MOPS (pH 7.2) and equilibrating against 15% PEG 3350, 4.2% MPD, 18 mM MOPS, 24 mM NaCl. They were cryoprotected in 25% PEG3350, 24 mM NaCl, 18 mM MOPS (pH 7.2) and 20% MPD. Data from a single crystal were collected at 100K to 2.3 Å resolution at the Cornell High Energy Synchrotron Source (beamline A1a, λ = 0.9764 Å). Data were reduced with the software package HKL version 1.98.1⁴³. There is one molecule in asymmetric unit. The structure was solved by molecular replacement method using AMoRe⁴⁷ and the previous scallop S2 structure (pdb code: 1NKN) was used as model. The structure was rebuilt in O version 8.0⁴⁶ and refined with CNS_SOLVE version 1.0⁴⁵. Among the different crystal forms, this structure shows the best order at the N terminus (see text and Table 1 legend). This region in the C2 space group appears to be stabilized by hydrophobic contacts between symmetry related B chains involving proline 835 and leucine 837. This structure has been deposited into the Protein Data Bank (PDB code: 3BAS).

Melting temperature determination

Circular dichroism spectroscopy was applied to compare thermal stability of scallop S2 near the head-rod junction to that from other regulated and non-regulated myosins (Fig. 3b). Fragments of equal length were used to make the comparison. Chimeric S2 fragments of mouse skeletal muscle myosin (Myh4, GenBank Identification 73921192), rat cardiac muscle myosin (Myh6, GI: 127741) and chicken gizzard smooth muscle myosin II (GI: 3915778), corresponding to “S2N51” were produced as for scallop “S2N51”¹⁹. Note that without the leucine zipper, these short S2 fragments do not dimerize^{27; 28}. Temperature denaturation experiments were performed on a JASCO J720 spectropolarimeter in 20 mM phosphate buffer at pH 7.5 with 50 mM NaCl and final protein concentration of 0.5 mg/ml. Samples were heated with a constant 1 K/min rate, and the ellipticity was monitored at the 222 nm minimum of the α -helical spectrum. Curves were analyzed for a two-state transition between the folded coiled-coil dimers and unfolded monomers and apparent melting temperatures (T_m) were calculated as previously described⁴⁸. All constructs showed spectra typical for α -helical coiled-coil proteins with double minima at 208 and 222 nm and a ratio of $\theta_{222}/\theta_{208}$ above 1. Thermal unfolding of all S2 fragments were completely reversible as expected for coiled-coil dimers (not shown).

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Fig. 1. Overview of the S2N51 structure and its critical location in the myosin II dimer

A) Schematic diagram of myosin II in an on-state conformation **B)** Schematic diagram of an expanded view of the construct crystallized in the present study. **C)** Ribbon diagram of the best ordered structure of S2N51, crystallized in the monoclinic C2 space group. Grey indicates C-terminally attached GCN4 leucine zipper and N-terminally attached tetrapeptide tag. S2N51 is colored by temperature factor (see fig. 2). Box indicates region immediately adjacent to the head-rod junction and described in more detail in figures 2 and 3. **D)** Schematic diagram the off-state conformation of regulated myosin II (adapted from ³⁴).

Fig. 2. The atomic structure of scallop S2N51 is now visualized, in one of the chains of each of the two monoclinic crystal forms, all the way back to its N terminus

This is possible because of the improved order of the peptide in these crystal forms. In this connection note that the pairwise rms deviation for residues 846-885 is 0.4-0.8 Å² among the coiled coils in the monoclinic crystals but is 1.1 Å² among those in the orthorhombic crystal. S2N51 is color coded in a rainbow ramp by temperature factor, where deep blue represents well-ordered regions (temperature factor at or below 20 Å²) and deep red is poorly ordered (temperature factor at or above 100 Å²). Region in oval is magnified in Fig. 3a. Locations of selected residues are indicated by their scallop myosin heavy chain residue numbers (bold text and underline indicate coiled-coil core residues that are visible in both chains).

Fig. 3. Diminished stability of the N-terminal region of the S2 coiled coil in REGULATED relative to non-regulated myosins

A) The structure of residues 835-837 in the scallop S2N51 peptide adopts a relatively extended non-helical conformation, in the two (of ten) examples where this region is ordered enough to be seen. (Note also different orientations of this segment relative to the coiled-coil axis in the two cases). Residues 835-837 however do adopt a helical conformation in the regulatory domain structure of scallop¹⁴ (as well as of *Physarum polycephalum*²³ and in some of the scallop S1 structures). Here, a knob-into-hole type of interaction of leucine 837 from the heavy chain (blue) with RLC helix A (green) appears to help stabilize this segment of the heavy chain, including proline 835, in a helical conformation. These residues are also helical in the human

cardiac S2 (delta) structure, even in the absence of an RLC. A nearby pair of *d-e'* salt links appears to stabilize the interactions of the coiled coil's two helical chains²⁰. **B)** Thermal denaturation experiments (see Methods) indicate lower apparent melting temperatures for S2 chimeras from regulated scallop muscle (▼) and chicken gizzard smooth muscle (λ) myosins than from non-regulated mouse skeletal muscle (◆) and rat cardiac muscle (■) myosins. **C)** Sequence alignment of different myosin II heavy chains shows a decreased number of potential interhelical salt links near the N-terminus of S2 in regulated myosins. Residues that are in position to participate in *g-e'* and *d-e'* interhelical salt links are underlined and are indicated by red and violet, respectively. The alignment also shows a distinct boundary between conserved (shading indicates identity to *Argopecten irradians* sequence) and variable regions starting at residue 838; Note that 824,826,829,830,833, and 837 (starred) are post-hook heavy chain residues of the lever arm that are highly buried by interactions with the RLC¹⁴, perhaps explaining their high sequence conservation. The myosin II isoforms displayed are as follows: (1) striated adductor muscle of the bay scallop *Argopecten irradians* (GenBank Identification 5612); (2) ventral siphon of the squid *Loligo pealei* (GI 3252880), (3) chicken gizzard muscle (GI 3915778); (4) human bladder and esophagus muscles (GI 13432177), (5) human cardiac muscle (beta myosin, GI 83304912); (6) rat cardiac muscle (alpha myosin, GI 127741), (7) mouse skeletal muscle (GI 73921192).

Figure 4. Diminished axial-stagger asymmetry in the N-terminal region of scallop S2 (red solid lines) relative to human cardiac S2 (blue dashed lines)

Axial staggering may therefore not be a general inducer of non-equivalence of S1 heads in off-state structures of myosin (see Discussion). The difference between these isoforms in the axial-staggering between the helices is most extensive for those structures (darkest shades) with the highest order nearest the N-terminus. Stagger at any residue is calculated from output of LSQAB⁴⁹ from the CCP4 program suite⁵⁰, in which 7-residue-long segments of the two helices of a coiled coil are fitted. The axial staggers are calculated for the N-terminal regions of five crystallographically independent scallop S2 coiled coils (that from the C2 space group and the two coiled coils from each of the P2₁2₁2₁ and P2₁ space groups) and of three crystallographically independent human cardiac S2 coiled coils (one wild type and two coiled coils in which a residue outside the measured region was mutated; (see 20).

TABLE 1

Crystallographic Statistics

Unit Cell:			
Space group	P2 ₁	C2	P2 ₁ 2 ₁ 2 ₁
a (Å)	65.8	123.3	51.9
b (Å)	40.2	44.9	72.7
c (Å)	77.4	39.9	104.2
alpha (°)	90.0	90.0	90.0
beta (°)	111.5	95.2	90.0
gamma (°)	90.0	90.0	90.0
Number of molecules/a.u.	2	1	2
Crystallization temperature	4°C	4°C	4°C [¶]
Data Collection			
Resolution Limits (Å):	30→1.95	50→2.3	30→3.0
Measured reflections	83110	27746	47546
Unique reflections	27340	8283	7779
Completeness, %	98.5 (100.0) [*]	84.9 (53.6) [*]	93.5 (72.6) [*]
R _{merge} [†]	0.052 (.103) [*]	.082 (.245) [*]	.071 (.37) [*]
Collection temperature	100K	100K	100K
Refinement[¶]			
Resolution Limits (Å)	30-2.3	20-2.3	30-3.0
No. of reflections (work/free)	15131/1704	7394/ 878	7229/387
Sigma cutoff	0	0	0
R _{cryst} [‡] /R _{free} [§]	0.253/ 0.299	0.247/ 0.286	0.233/0.284
No. of protein/ water atoms	2695/ 99	1386/ 59 ^{**}	2519/0
rms bond lengths (Å)/ angles (°)	0.007/ 0.864	0.006/ 0.880	0.01/1.5

[¶]See 19 for statistics, to 2.5-Å resolution, from a similar P2₁2₁2₁ crystal grown at 16°C (and data collected at 100K).

^{*} Values for select high-resolution bins (C2: 2.38 - 2.30Å, P2₁: 2.46-2.31 Å, P2₁2₁2₁: 3.11-3.0) are shown in parentheses.

[†] $R_{\text{merge}} = \sum |hkl|I - \langle I \rangle / \sum I_{hkl}$.

[‡] $R_{\text{cryst}} = \sum |hkl| |F_{\text{obs}}| - |F_{\text{calc}}| / \sum |hkl| |F_{\text{obs}}|$, where F_{calc} and F_{obs} are calculated and observed structure factor amplitudes, respectively.

[§] R_{free} is the same as R_{cryst} except that the summation is over the randomly selected “free” reflections excluded from the refinement.

^{**} In addition, in the C2 space group, one heteroatom, modeled as an iodide, was included in the final refinement procedures, to reflect a peak of density found in an apolar environment near the two N-termini of symmetry related molecules (directly on a two-fold axis). Although not included in the precipitant conditions in this space group, the iodide may have been introduced as a contaminant. Inclusion in the refinement reduced the R-factors by ~ 2%.